

MSc in Nutrition & Dietetics

**Resveratrol-induced cell death in leukaemia cells: the  
effect of Hsp72 expression and combination  
treatments with TRAIL and ethanol**

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## **Abstract**

Resveratrol, a natural phytoalexin found in grapes and red wine, displays anti-cancer activities through a variety of mechanisms that include the induction of cancer cell apoptosis. Although high concentrations may be needed for the efficacy of resveratrol alone, the compound shows promise as a potent sensitizer of the apoptotic effect of other anti-cancer agents, including death ligand TRAIL. Intracellular heat shock proteins (Hsps) are frequently up-regulated in cancer cells, conferring resistance to apoptosis. Modulation of these proteins may overcome the resistance and increase efficacy of anticancer therapies.

In this study, resveratrol caused significant dose-dependent apoptosis or necrosis in the lymphoid and myeloid leukaemia cell lines Jurkat and U937 at 50 $\mu$ M and above. Combination treatments with sub-lethal concentrations of ethanol or TRAIL showed synergistic effects on the dose-dependent cell death, such that significant apoptosis was achieved at 25 $\mu$ M resveratrol. Treatment with all compounds together showed the greatest efficacy with significant apoptosis occurring at 12.5 $\mu$ M resveratrol, but also greater toxicity demonstrated by a shift from apoptosis to necrosis occurring at 25-50 $\mu$ M as opposed to 100-200 $\mu$ M resveratrol in the other combination treatments.

Low concentrations of resveratrol that were unable to induce apoptosis caused a significant increase in intracellular Hsp72, whilst intracellular Hsp72 was unchanged or reduced at higher concentrations. Increasing intracellular Hsp72 expression in cells

with a mild heat shock prior to resveratrol treatment caused increased resistance to resveratrol-induced cell death at 50-200 $\mu$ M. It was not possible to show that lowering intracellular Hsp72 with ethanol treatment increased sensitivity to resveratrol-induced cell death. It was shown for the first time that resveratrol increased surface expression of Hsp72 at all concentrations, highlighting the potential for an additional anti-cancer effect *in situ*.

The results indicate that combination treatments with resveratrol are promising for use in anticancer therapy, and manipulation of intracellular Hsp72 expression may modulate the efficacy of resveratrol-induced apoptosis. Further investigations are recommended into the clinical relevance of resveratrol, further synergistic compounds, and the effects of reducing intracellular and increasing surface Hsp72 levels.

## **Declaration of original work**

I hereby declare that work contained herewith is original and is entirely my own work (unless indicated otherwise). It has not been previously submitted in support of a Degree, qualification or other course.

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## 1. Introduction

### 1.1. *Resveratrol and its health-beneficial properties*

Resveratrol (3,5,4'-hydroxystilbene) is a polyphenolic compound produced in a number of plant species in response to stress. It protects plants by blocking the proliferation of pathogens (Delmas et al., 2006). Resveratrol is thought to be particularly abundant in grapes and wine, and is also found in mulberries, cranberries, peanuts, and plants of the *Cassia quinquangulata* family (Gescher and Steward, 2003). As a constituent of grape skin, its concentration reaches 10–20mM in red wine (Manna et al, 2000). Resveratrol is believed to be found in sufficiently high concentrations in dietary components to provide a potential impact on human health (Baur and Sinclair, 2006). Both *trans* and *cis* isomers of resveratrol exist, but as the *trans* isomer is much more common it is this form that is most commonly considered, and referred to throughout this paper (Signorelli and Ghidoni, 2005).

Efforts to identify dietary compounds with health-promoting activity intensified when the link between diet and pathogenesis was established (Hsieh et al, 2008). Interest in resveratrol has increased concomitantly with the discoveries of many pronounced pharmacological effects displayed in different experimental models. A strong collection of evidence from a large number of epidemiological, animal and *in vitro*

studies suggests that resveratrol exhibits a wide range of beneficial effects for health (Bernhard et al., 2000).

Resveratrol has been shown to be protective at the cellular level against various toxicities and neurodamage, and in a larger/systemic context against cancer, cardiovascular disease and age-related neurodegenerative disorders (Han et al., 2006). It has been widely hypothesised that resveratrol plays an important part in the reported benefits of wine consumption and reduced mortality from cardiovascular disease observed in the French paradox<sup>1</sup>. This hypothesis is based on research demonstrating that resveratrol is a powerful antioxidant and anti-inflammatory agent, prevents platelet aggregation and coagulation, modulates arachidonic acid metabolism, lipid metabolism and prevents LDL oxidation (Bernhard et al., 2000).

In 1997, resveratrol was also shown to be an effective anti-cancer agent in all stages of carcinogenesis (Jang et al., 1997), and this propelled the interest in the compound as a chemopreventive and chemotherapeutic agent. Its protective effects by preventing aberration of normal cells have now been well established; however, resveratrol has also been shown to be cytotoxic towards transformed cells (Delmas et al., 2006). Thus, the effects of resveratrol vary between pro-survival and pro-death mechanisms, seemingly dependent on what is required to promote the health of the particular tissue (Signorelli and Ghidoni, 2005).

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<sup>1</sup> The French paradox is the observation that cardiovascular disease is reported as cause of death in relatively lower numbers in the French compared with other Western populations, despite the French diet being high in saturated fats, a known contributing factor to cardiovascular disease. Their diets are also reported to comprise relatively high levels of red wine.

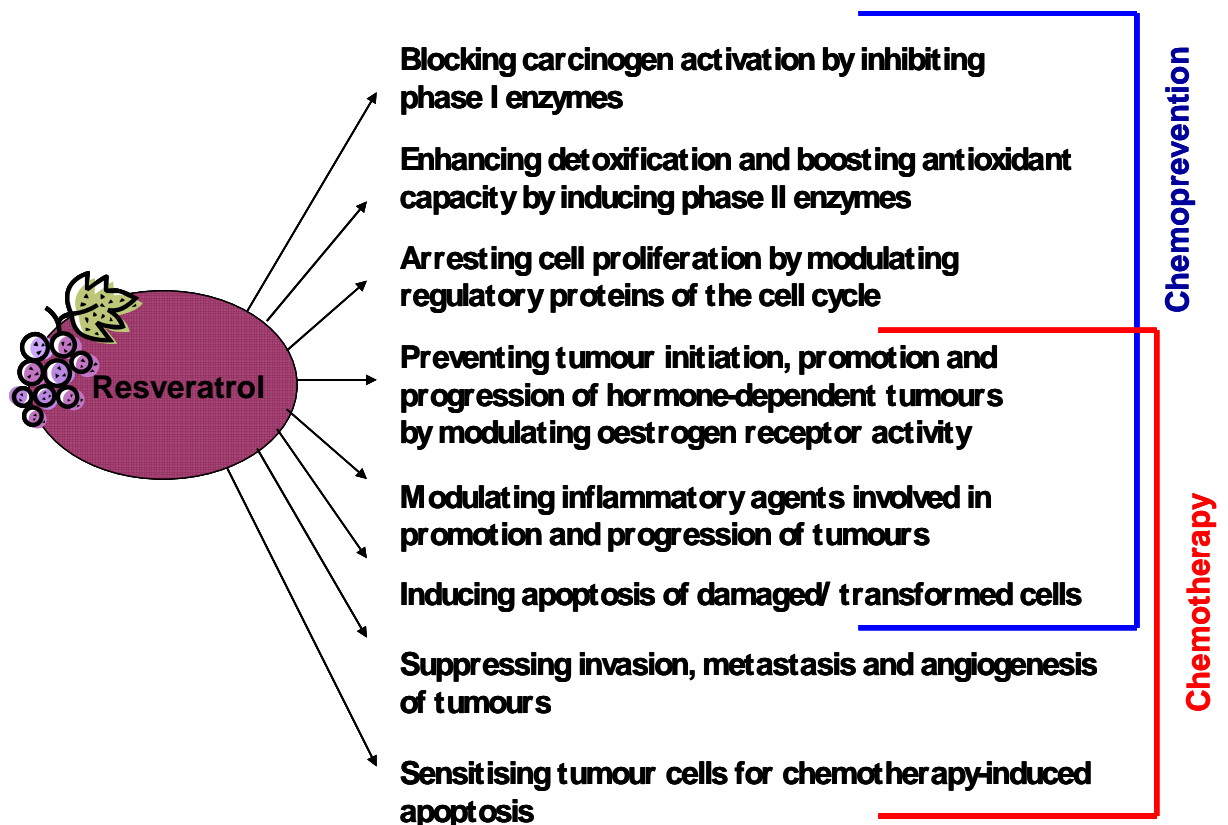
Resveratrol is a lipophilic compound that can cross the plasma membrane of cells, and has been shown to enter cells via passive diffusion as well as carrier-mediated transport (Lançon et al., 2004). Resveratrol also acts at the membrane, binding to specific receptors, e.g. the oestrogen, insulin-like growth factor-1 and epidermal growth factor receptors, and various other membrane sites (Han et al., 2006). Research is being conducted to elucidate specific protein targets for resveratrol. It has been observed to interact with several targets on the cell membrane and intracellular environment, which have been termed resveratrol-targeting proteins (RTPs; Hsieh et al., 2008).

The various beneficial effects of resveratrol are attributed not only to its powerful antioxidant nature, but more importantly to this ability to interact with a wide range of cellular targets and thus, modulate various signal transduction pathways (Han et al., 2006). For example, resveratrol may protect against toxicity in hippocampal cells by rapidly activating the protein kinase C pathway (Han et al., 2004) and induce apoptosis of various tumour cell lines by inhibiting the PI3 kinase/AKT or MAPK pathways (Aziz et al., 2006; Fulda and Dehtain, 2006). The diversity of effects displayed are thought to be dependent on cell type, cellular condition, molecular setting and concentration of resveratrol (Hsieh et al., 2008; Signorelli and Ghidoni, 2005). Hsieh et al. (2008) suggest that the diverse effects and broad concentration-dependence of resveratrol may be explained by qualitative and quantitative differences in type and level of RTPs in different cells. Many different molecular mechanisms displayed by

resveratrol have been reported, but further research is still required to determine mechanisms displayed in different cellular and molecular settings.

### ***1.2. Anti-cancer properties of resveratrol***

Jang et al. (1997) first highlighted the promising anti-cancer properties of resveratrol by demonstrating its activity against all three stages of carcinogenesis: initiation, promotion and progression. They demonstrated that resveratrol is able to inhibit initiation and promotion of hydrocarbon-induced skin cancer and the progression of breast cancer in mice. Further research continues to show promising roles for resveratrol in more advanced stages of cancer, most recently as a possible compound to enhance the efficacy of the body's natural defences and anti-cancer drugs (Delmas et al., 2006).



**Figure 1.1.** *Resveratrol displays a variety of anti-cancer mechanisms which are chemopreventive, chemotherapeutic, or both. Adapted from Kundu and Surh (2008).*

As illustrated in figure 1.1, resveratrol may exert its anti-cancer activities through different mechanisms that may be classed as chemoprevention, chemotherapy or encompass both.

- A major chemopreventive effect results from the ability of resveratrol to modulate phase I and II enzymes which are involved both in the activation and detoxification of carcinogens. Resveratrol has been shown to inhibit phase I enzymes in the metabolism of xenobiotics, thus preventing activation of carcinogenic forms of chemicals. In addition, resveratrol has been shown to induce phase II

antioxidant/detoxification enzymes, thereby speeding up the excretion of potentially carcinogenic agents and boosting antioxidant activity (Signorelli and Ghidoni, 2005; Kundu and Surh, 2008).

- Another chemopreventive effect results from the ability of resveratrol to modulate regulatory proteins of the cell cycle, thus causing cell cycle arrest and inhibition of abnormal proliferation. Several studies have shown that resveratrol causes growth arrest of different tumour cell lines at different stages of the cell cycle. The growth arrest may be irreversible and progress to apoptosis (Signorelli and Ghidoni, 2005).
- Resveratrol has been shown to modulate mediators of inflammation e.g. cyclooxygenase-2 (COX-2) and nitric oxide (NO) which are involved in promotion and progression of cancers by stimulating cell growth and migration (Kundu and Surh, 2008). Thus, the anti-inflammatory properties of resveratrol contribute to its chemo-preventive and -therapeutic effects.
- Resveratrol is a selective modulator of oestrogen receptor activity, a property that may prevent tumour initiation, promotion and progression of hormone-dependent tumours (e.g. breast, prostate, lung and colon; Signorelli and Ghidoni, 2005).
- Resveratrol has been shown to induce apoptosis in several different tumour cells *in vitro* as well as in chemically induced and transplanted tumours in mice (Garvin et al., 2006; Kalra et al., 2008; Clément et al., 1998; Bernhard et al., 2000; Dörrie et al., 2001; Delmas et al., 2003). Several different apoptotic mechanisms of resveratrol have been reported within both the extrinsic and intrinsic apoptotic pathways, and may vary between different cell types. A large collection of evidence suggests that resveratrol-induced apoptosis is linked to its ability to:



- modulate anti- and pro-apoptotic proteins and pathways. For example, resveratrol has been shown to deplete ‘inhibitor of apoptosis proteins’, e.g. survivin (Hayashibara et al., 2002). High levels of survivin have been found expressed in the majority of human cancers, and depletion of survivin by resveratrol has been found to sensitise such cells to apoptosis. Resveratrol also modulates intracellular signalling pathways involved in apoptosis, e.g. by inhibiting the anti-apoptotic PI3K/AKT and MAPK pathways in several tumour cell lines (Fulda and Debatin, 2006).
- regulate gene expression of proteins involved in apoptosis, e.g. by modulating transcription factors, inhibiting NF $\kappa$ B pathways and upregulating p53-responsive genes (Fulda and Debatin, 2006)
- activate death receptor signalling on the cell surface, e.g. CD95-CD95L signalling (Clément et al, 1998), by redistributing death receptors on the cell membrane sensitising cells to apoptosis (Delmas et al., 2004)
- modulate several other cellular mechanisms, e.g. sphingolipid signalling and drug transporters causing tumour cells to become sensitised to apoptosis (Signorelli and Ghidoni, 2005)
- Resveratrol has been shown to suppress invasion, metastasis and angiogenesis of tumours by inhibiting multiple proteins and signalling cascades involved in these processes (Kundu and Surh, 2008). For example, resveratrol has been shown to inhibit VEGF-induced angiogenesis (Lin et al, 2003). Resveratrol has also been shown to sensitise tumour cells to apoptosis induced by death receptors and anticancer drugs and therapies (Fulda and Debatin, 2004; Sun et al., 2002; Kubota

et al., 2003; Jazirehi and Bonavida, 2004; Zoberi et al., 2002; Fulda and Debatin, 2005; Delmas et al., 2004).

- For comprehensive reviews on the mechanisms behind resveratrol's anti-cancer effects see Kundu and Surh (2008), Signorelli and Ghidoni (2005) and Fulda and Debatin (2006).

### **1.2.1. Resveratrol-induced apoptosis in anti-cancer therapy**

Despite advances in anti-cancer therapies, resistance of tumour cells to treatment and relapse of the disease have proved to be major setbacks to survival rates of sufferers. Certain leukaemias have proved particularly resistant to anticancer therapy demonstrating the need for new and more effective treatments (Cecchinato et al., 2007). Resveratrol has been suggested to be a promising anti-leukemic agent (Gautam et al., 2000). Several studies have demonstrated the anti-proliferative and apoptotic effect of resveratrol in various myeloid (U937, HL60, THP-1, KCL22, and K562) and lymphoid (Jurkat and WSU-CLL) leukaemia cell lines (Clément et al., 1998; Ferry-Dumazet et al., 2002; Park et al., 2001; Su et al., 2005; Dörrie et al., 2001; Manna et al., 2000).

Pathways involved in resveratrol-induced cell death have been characterised in leukaemia cells previously. Apoptosis through the Fas-FasL pathway has been demonstrated (Clément et al., 1998) as well as through the Fas-independent,

mitochondria/caspase-9 pathway in cells that are resistant to Fas signaling (Dörrie et al., 2001). Su et al. (2005) demonstrated that in HL-60 cells resveratrol induced apoptosis through the Cdc42/apoptosis signal-regulating kinase 1/c-Jun N-terminal kinase/FasL signaling cascade. Resveratrol has also been observed to down-regulate anti-apoptotic proteins (e.g. survivin, iNOS and Bcl-2; Fulda and Debatin, 2005; Athar et al., 2007) and significantly suppress interleukin activation of nuclear factor kappa- $\beta$  (NF $\kappa$ B; Athar et al., 2007) in leukaemia cells *in vitro*. Manna et al. (2000) observed that resveratrol caused suppression of NF $\kappa$ B induction by inhibiting activation of mitogen-activated protein kinase (MAPK) kinase and c-Jun terminal kinase in Jurkat, U937, HeLa and H4 cells, and that resveratrol abrogated TNF- $\alpha$  cytotoxicity. It has also been proposed that resveratrol increases susceptibility of K562 leukaemia cells to apoptosis by interfering with Hsp70 expression (Chakraborty et al., 2008).

Whilst the protective anti-inflammatory and anti-proliferative effects of resveratrol are believed to affect normal and transformed cells, the apoptotic effect of resveratrol has been highlighted as promising for anticancer therapy by potentially being cancer cell specific (Signorelli and Ghidoni, 2005). Several studies have reported that resveratrol causes apoptosis of cancer cells whilst being non-toxic to equivalent normal cells. Clément et al. (1998) reported that 16-32 $\mu$ M resveratrol induced apoptosis in HL60 human leukaemia cells after 24 hours treatment, but up to 72 hours incubation with the same concentrations showed minimal toxicity to peripheral blood lymphocytes (PBLs). Similarly, Dörrie et al. (2001) reported that resveratrol caused  $\geq 80\%$  cell death of several leukaemia cell lines whilst no significant toxicity on peripheral blood

mononuclear cells was detected. Gautam et al. (2000) reported resveratrol-induced apoptosis of human leukaemia cell lines U937 and HL-60, but no apoptotic effect on normal hematopoietic progenitor cells. Resveratrol has also been shown to induce apoptosis of human prostate cancer cells (LNCaP) whilst not affecting normal prostate epithelial cells (Aziz et al., 2006).

However, despite several studies suggesting that the apoptotic effect of resveratrol is cancer cell specific, Ferry-Dumazet et al. (2002) reported a general toxicity of resveratrol on normal and leukaemic haematopoietic cells. The team studied the toxicity of resveratrol on several different haematopoietic cells and confirmed previous reports of minimal toxicity to inactivated PBLs, but reported resveratrol-induced apoptosis of several other normal cycling haematopoietic cells. They suggested that this toxicity makes resveratrol inappropriate for use as a primary anti-leukaemic agent (Ferry-Dumazet et al., 2002).

Different cell types vary in sensitivity and pharmacological outcome to resveratrol. Whilst nanomolar concentrations may be effective in some cells, higher concentrations are required for efficacy in other cells (Signorelli and Ghidoni, 2005). Since relatively high concentrations of resveratrol may be required for efficacy and toxicity to normal cells has been reported, resveratrol may have greater value combined with other anticancer therapies. Low concentrations of resveratrol have proved highly effective at sensitising various tumour cells to apoptosis induced by death ligands and anticancer drugs (Fulda and Debatin, 2004; Fulda and Debatin, 2005; Delmas et al., 2004; Sun et

al., 2002; Kubota et al., 2003; Jazirehi and Bonavida, 2004). Synergistic combination treatments may enhance cancer cell death and reduce toxicity and undesirable side effects experienced when high concentrations of single compounds are used.

### ***1.3. Apoptosis***

Apoptosis is often dysregulated in disease. Hyperproliferation and cancer results when apoptosis is prevented, whereas excessive apoptosis causes tissue damage/loss in ischemic diseases and neurodegenerative disorders (Fulda and Debatin, 2006). Cancer cells persist by continuing to evade the normal process of apoptosis. This requires deregulation of several proteins involved in cell survival, e.g. inhibition of pro-apoptotic proteins and activation of anti-apoptotic/survival proteins.

Most cytotoxic cancer therapies operate by stimulating apoptosis (Fulda and Debatin, 2006; Johnstone et al., 2002). Thus, their efficacy depends on the ability to target functional apoptotic pathways. However, many tumours prove resistant to treatment, even when aggressive, due to the deregulation of apoptotic signalling pathways (Fulda and Debatin, 2006). This is most deeply featured in advanced cancer cells and reduces or prevents drug efficacy. Methods to enhance the apoptotic effect of anti-cancer agents are required to target tumour resistance and develop more effective treatments.

Apoptosis is a highly regulated and characteristic process of cell death essential for tissue homeostasis (Moulin & Arrigo, 2006). The process involves a caspase cascade in which activator caspases activate effector caspases, which fragment cellular components and cause internal cellular destruction. Apoptosis can occur via two pathways: 1) the intrinsic pathway in which intracellular stress signals target the mitochondria and cause release of cytochrome c, and 2) the extrinsic pathway in which

death receptors are activated by ligation on the cell surface and form a death-inducing signalling complex (DISC). The activator caspase in the intrinsic pathway, caspase-9, is formed upon cytochrome c release from stressed mitochondria and the formation of an apoptosome complex leading to caspase-3 activation (Fulda and Debatin, 2005). In the extrinsic pathway, death receptor stimulation results in the recruitment of an adaptor molecule (e.g. Fas-associated death domain [FADD]) and the formation of the DISC activates caspase-8 (Fulda and Debatin, 2005; Clemons and Anderson, 2006). Cross talk exists between the two pathways, and extrinsic death signals can lead to activation of the intrinsic pathway via members of the Bcl-2 family which cause release of cytochrome c upon cleavage by caspase-8 (Fulda and Debatin, 2005).

It is believed that death receptor ligands bind to pre-associated trimers of their receptors (Delmas et al., 2004) and that two ligands bound to a hexameric receptor complex is required to induce apoptosis. Strong DISC formation and more efficient apoptosis signalling is believed to result from the clustering of multiple receptor trimers within membrane microdomains (lipid rafts).

Death receptors are members of the tumour necrosis factor (TNF) receptor superfamily, and include TNF- $\alpha$ , CD95 (APO-1/Fas) and TNF-related apoptosis-inducing ligand (TRAIL; Fulda and Debatin, 2006; Clemons and Anderson, 2006). TRAIL binds to death receptors DR4 and DR5 to induce apoptosis, but may also bind to decoy receptors DcR1 and DcR2 which do not stimulate apoptosis. TRAIL is a promising agent for anti-cancer therapy because it has been shown to induce apoptosis

specifically of cancer cells and display little toxicity (Psahoulia et al., 2007). Whilst systemic toxicity after administration of other death ligands (CD95L and TNF $\alpha$ ) has been reported, administration of TRAIL has not produced such toxicity in mice or non-human primates (Fulda and Debatin, 2005). Unfortunately, cancer cells can also display resistance to TRAIL-induced apoptosis, possibly due to expression of decoy receptors (Clemons and Anderson, 2006).



#### ***1.4. Heat Shock Proteins***

Altered heat shock protein (Hsp) expression has been reported in a wide range of tumour cells and over-expression is believed to confer resistance to apoptosis (Jolly and Morimoto, 2000). Over-expression of Hsps is a common feature of cancer cells and has been associated with resistance to therapy and poor prognosis of the sufferers (Clemons and Anderson, 2006; Lanneau et al., 2008).

Hsps are a highly conserved class of proteins that act as molecular chaperones and proteases within the cell and are expressed at elevated levels in response to stress to counteract toxicity and cell death. They serve essential maintenance and protective roles under normal conditions (Garrido et al., 2001; Multhoff, 2007). Under stress, their roles are expanded and Hsps include anti- and pro-apoptotic proteins that act at various points in the apoptosis pathways. “Their expression level can determine the fate of a cell in response to a death stimulus” (Garrido et al., 2001). Hsps are induced by most forms of cellular stress. Their induction is often referred to as a heat shock response or stress response. There are six major families of Hsps, traditionally named after their molecular size: Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and small Hsps (Jolly and Morimoto, 2000). Kampinga et al. (2009) have recently proposed new guidelines for the nomenclature of human Hsps. Although this paper will use the traditional nomenclature, the author realises the value of using the new nomenclature in future work.

#### **1.4.1. Induction of Hsp expression**

Each Hsp family includes members with constitutive or inducible expression, and that reside in different cellular compartments. Expression of inducible proteins is quickly up-regulated by appropriate stress or apoptotic stimuli (Sõti et al., 2005). The induction occurs when heat shock factor (HSF)-1 is activated and translocates to the nucleus to act as a transcription factor. Under normal conditions it is maintained inactive by the binding of Hsps, which dissociate from and activate HSF-1 when they become occupied by increases in damaged proteins (Sõti et al., 2005). However, Hsps are present at abnormal levels in many disease states, including several cancer cell types, without any evidence existing for increased levels of damaged proteins to explain the effect. Certain compounds, e.g. curcumin, will induce a heat shock response in transformed cells but not normal cells (Khar et al., 2001). It is now known that different types of stresses can induce different expression patterns of Hsps, suggesting that several mechanisms to regulate Hsps expression exist (Sõti et al., 2005). Thus, not only increased levels of damaged proteins are able to induce Hsp expression and alternative signals for induction have been proposed.

There is growing interest in the concept that the cell membrane acts as a sensor for stress and can induce Hsps expression. It has been shown that stress-induced membrane alterations transmit signals to induce Hsp expression (Carratù et al., 1996; Horváth et al., 1998; Vigh et al., 2007). Stress-induced alterations include changes in membrane fluidity and composition, which often relate to changes in membrane

microdomains termed 'lipid rafts'. There is mounting evidence that hyperfluidization of the cell membrane induces the Hsp response as a result of reorganisation of the lipid rafts (Balogh et al., 2005; Nagy et al., 2007; Vigh et al., 2007) suggesting that agents that cause such membrane alterations may induce the heat shock response.

#### **1.4.2. Hsps and apoptosis**

Hsps have been found to play an important protective role against apoptosis by interfering with caspase activation. Either of Hsp27, Hsp60, Hsp70, and Hsp90 have been found to inhibit caspase activation and prevent apoptosis when over-expressed, and increase cellular sensitivity to apoptotic stimuli when depleted (Lanneau et al., 2008). Hsp27 and Hsp70 are believed to be more strongly anti-apoptotic and are implicated in carcinogenesis. Elevated cytosolic levels of these proteins have been shown to increase the tumorigenic potential of rodent cells in experimental models (Garrido et al., 2001).

The Hsp70 family is the best studied class of Hsps and the most conserved. It comprises at least 11 genes in humans, including the constitutively expressed Hsc70 and the inducible Hsp72 that are mainly found in the cytosol, the mitochondrial mtHsp70 and the endoplasmic reticulum-localised Grp78 (Lanneau et al., 2008). Hsp72 is believed to be one of the most powerful anti-apoptotic proteins by being able to block almost all identified cell death pathways. High expression of Hsp70 alone has

been shown to prevent apoptosis from being induced by a variety of stresses (Clemons and Anderson, 2006), whereas elimination of Hsp70 expression with antisense oligonucleotides has been shown to result in apoptosis and cell growth arrest of cancer cells (Jolly and Morimoto, 2000).

### **1.4.3. Dual role of Hsps**

In recent years, there has been growing evidence of a dual role of Hsps. Whilst exhibiting strong cytoprotective properties in the intracellular environment, membrane-bound and extracellular Hsps have been shown to display immunogenic properties to the extent that Hsp70 expression at the plasma membrane can facilitate elimination of tumour cells by the immune system (Multhoff, 2007). Particularly members of the Hsp70 and Hsp90 families have been observed in the extracellular environment. Hsp70 has been shown to be frequently present on the plasma membrane of several cancer cells including leukemia cells, with no membrane localisation of Hsp70 being found in the corresponding normal tissues (Schmitt et al, 2007).

Elevated levels of Hsp70 and Hsp90 have been detected in the medium of tumour cell lines and in antigen presenting cells, as well as in the serum of cancer patients (Schmitt et al., 2007; Multhoff, 2007). Serum levels may be the result of proteins expelled in apoptosis and necrosis. However, this mechanism is unlikely to account for the magnitude detected and there is increasing evidence for an active secretion process of

Hsps from viable tumour cells, despite Hsps not containing leader peptides. Several research groups have observed active release of Hsp70 from the cytosol to the plasma membrane. It has been suggested that Hsp70 release may occur via a non-classical protein secretion pathway involving lipid rafts (Hunter-Lavin et al., 2004), and active release from viable tumour cells via exosomes has been reported by Gastpar et al. (2005). Stress stimuli have shown to be capable of increasing active release of Hsp70 from these cells (Schmitt et al., 2007).

The immunogenic roles of extracellular Hsps have been shown to be varied and play an important part in modulating the innate and adaptive immune responses, with the roles of Hsp70 being the best studied. Different functions of Hsp70 include stimulating the release of chemokines, proinflammatory cytokines and nitric oxide, facilitating receptor-mediated endocytosis and processing and presenting peptides on MHC class I molecules (Schmitt et al., 2007). Tumour cells that present Hsp70 on the cell surface have been shown to stimulate activation by natural killer (NK) cells. (Schmitt et al., 2007).

Treatment of cells with various reagents/treatments e.g. hyperthermia has been shown to increase the expression of membrane-bound Hsp70 on the surface of tumour cells and thus, increase sensitivity to NK cell-mediated killing. The effect was tumour cell-specific for some treatments (Gehrmann et al., 2002). Gehrmann et al. (2004) demonstrated this effect using anti-inflammatory drugs including inhibitory agents of COX1/2, and suggested that the effect is a biological rationale for combining such drugs

with immunotherapy in cancer therapy. Resveratrol is an anti-inflammatory inhibitor of COX1/2 and thus may stimulate a similar effect.

#### **1.4.4. Hsps and resveratrol in anti-cancer therapy**

Due to the important localisation-dependent roles of Hsps in tumour cell survival, agents that block the intracellular and enhance the extracellular functions of Hsps are believed to be of benefit in anticancer therapy. Either method has shown promising effects on tumour suppression. However, it has been proposed that both effects in combination would be most effective (Scmitt et al., 2007).

There are various and contradictory reports of the effect of resveratrol on Hsp70 expression in cancer cells. Chakraborty et al. (2008) reported that 40 $\mu$ M resveratrol induced apoptosis in myelogenous leukaemia K562 cells by suppressing intracellular Hsp70 expression, correlating with a downregulation of HSF-1 activity. Thus, resveratrol was able to significantly enhance the apoptotic effect of the anticancer drug 17-allylamino-17-demethoxygeldanamycin that is known to augment Hsp70 levels. However, other studies have found that resveratrol induces Hsp70 expression in other cell lines. Cardile et al. (2003) reported that lower concentrations of resveratrol (50-100 $\mu$ M) significantly raised Hsp70 levels in prostate cancer DU-145 cells, whilst the higher concentration (200 $\mu$ M) showed no difference in Hsp70 levels than control cells. Putics et al. (2008) reported that resveratrol induced the expression of Hsp70 in human

peripheral lymphocytes, monkey fibroblasts and human cervical cancer cell line HeLa, but did not demonstrate a reduction in Hsp70 levels at higher concentrations of resveratrol. They reported that the resveratrol-induced Hsp70 elevation synergized with mild-moderate heat stress to protect cells against severe heat stress. None of the studies investigated the cellular location of the induced Hsp70s.

#### **1.4.5. Combination treatments - Resveratrol and TRAIL and ethanol**

Resveratrol has been shown to sensitize cancer cells to cell death induced by anticancer agents AraC, 5-fluoro-uracil, paclitaxel and radiation therapy (Fulda and Debatin, 2004; Sun et al., 2002; Kubota et al., 2003; Jazirehi and Bonavida, 2004; Zoberi et al., 2002 in Delmas et al. 2004) as well as to TRAIL-induced apoptosis (Fulda and Debatin, 2005; Delmas et al., 2004). Relatively low concentrations of resveratrol (10-30 $\mu$ M) have been shown to be effective to produce a significant sensitization effect (Jazirehi and Bonavida, 2004; Fulda and Debtain, 2004; Delmas et al., 2004).

Several studies have linked resveratrol-induced apoptosis with modulation of death receptors. Clément et al. (1998) observed that resveratrol enhanced CD95L activity in HL60 and T47D cells, and suggested that resveratrol-induced cell death is CD95/Fas-signaling dependent. Delmas et al. (2004) demonstrated that resveratrol sensitised colon cancer cells to apoptosis induced by death receptor ligands (TNF, anti-CD95 antibodies, TRAIL) without increasing the number of death receptors on the cell

membrane but by inducing the redistribution of CD95, DR4 and DR5 into lipid rafts, which is believed to aid DISC activation. Quercetin, another dietary polyphenol in red wine, has also been shown to enhance TRAIL-induced apoptosis directly by accumulation of death receptors into lipid rafts (Psahoulia et al., 2007). Ethanol has been shown to be a potent sensitizer of colon cancer cells to TRAIL-induced apoptosis (Vaculová et al., 2004). The effect has not been directly linked with lipid raft modulation, but ethanol is known to be a membrane fluidizer and causes lipid raft clustering at relatively low concentrations (50 mM; Nourissat et al., 2008). Thus, it is feasible that lipid raft aggregation is also partly responsible for the sensitization effect of ethanol.

An important property for anticancer agents is the ability to overcome anti-apoptotic proteins. Whilst DNA-damaging cytotoxic drugs used to sensitise cells to death receptor-mediated apoptosis are unable to overcome the inhibitory effect of Bcl-2 overexpression observed in several tumour cell types (Micheau et al., 1997; Lacour et al., 2003), Delmas et al. (2004) showed that resveratrol is. Delmas et al. (2004) suggested that the difference is caused by an ability of resveratrol to assist in stronger DISC formation by the modulation of lipid rafts. However, Fulda and Debatin (2005) showed that resveratrol was unable to overcome overexpression of Bcl-2 and FADD-DN suggesting that these uncoupled the apoptotic effects of resveratrol (cell cycle arrest and surviving depletion) with the caspase cascade. This study used up to 30ng/ml TRAIL in Jurkat or SHEP cells whilst Delmas et al. (2004) used 100ng/ml in colon cancer cells, suggesting that the effect may be TRAIL dose dependent or cell type



specific (i.e. cells have differing sensitivities to TRAIL). Vaculová et al. (2004) showed that Bcl-2 or the mitochondrial pathway was not involved in the potentiation of TRAIL-induced apoptosis by ethanol, and proposed the involvement of other mechanism including modulation of anti-apoptotic signals involved in TRAIL resistance. Thus, it appears that although ethanol and resveratrol may have similar effects and both cause lipid raft aggregation, they may also differ in mechanisms to sensitize cells to apoptosis. It would be interesting to investigate whether a greater sensitization effect could be achieved by using the compounds in combination, particularly since ethanol is a constituent of red wine which has been proposed to be the most abundant source of resveratrol in the human diet.

Moderate consumption of alcoholic drinks, including red wine, has been suggested to have health beneficial properties (Opie and Lecour, 2007). However, the World Research Cancer Fund has also linked alcohol consumption with increased risk of cancer (Wiseman, 2008). Alcohol-free red wine may be a superior source of resveratrol for health. This makes sense in chemopreventive aspect. However, moderate amounts of ethanol has been shown to have some similar health beneficial effects to resveratrol, e.g. anti-inflammatory and immune modulating properties (Szabo et al., 2007) and ability to sensitize cancer cells to TRAIL (Vaculová et al., 2004). Since alcoholic red wine is more available and more commonly consumed, it would be interesting to investigate the effect of the combination of ethanol and resveratrol on cancer cells, as well as in combination with TRAIL.

### ***1.5. Bioavailability and Clinical Potential of Resveratrol***

There is interest in a number of different polyphenols in red wine for potential health benefits including protection against inflammation, atherosclerosis and cancer (Soleas et al., 2002). Soleas et al. (2002) used a mouse skin cancer model assessing initiation and promotion of cancer to compare the antitumorigenic activities of four common red wine polyphenols: (+)-catechin, quercetin, gallic acid, and *trans*-resveratrol. Whilst quercetin was the most effective at inhibiting tumour formation and resveratrol showed intermediate efficacy, the research team concluded that in terms of human consumption of red wine, *trans*-resveratrol may be the most effective anticancer polyphenol consumed. This is due to its relatively high concentration in red wine and much more efficient absorption by humans than the other polyphenols that displayed antitumorigenic activity, as reported by the same research team (Goldberg et al., 2003).

Goldberg et al. (2003) compared how efficiently *trans*-resveratrol, [+-]-catechin and quercetin were absorbed by healthy human males after oral consumption in three different media (white wine, grape juice and vegetable juice) and concluded that the absorption of resveratrol was the most efficient, and that the absorption was similar in aqueous and alcoholic media. However, the peak concentrations found in serum (10-40nmol/L) were too low to be compared with *in vitro* findings. All polyphenols were predominantly present in serum as glucuronide and sulfate conjugates so that the research team concluded that the literature regarding the effects of the polyphenols are irrelevant since they are absorbed as conjugates in the human body.

## ***1.6. Aims of the study***

Resveratrol has been shown to induce apoptosis in Jurkat cells and to a lesser extent in U937 cells (Park et al., 2001; Ferry-Dumanetz et al, 2002; Dörrie et al., 2001; Fulda and Debatin, 2004). The cell lines are suitable for the study as they have been shown to be relatively sensitive to resveratrol. Contradictory results exist on the effect of resveratrol on intracellular Hsp72, whilst the effect on extracellular/surface Hsp72 has not been investigated. Resveratrol has been shown to sensitise Jurkat cells to TRAIL-induced apoptosis, but the evidence is missing for U937 cells. There are no reports on the effect of resveratrol and ethanol in combination despite red wine being the largest source of resveratrol in the Western diet (Baur and Sinclair, 2006).

Suppression of intracellular Hsp72 expression is a promising mechanism to render cancer cells more susceptible to treatment. It is possible that resveratrol causes such a suppression, and if not, resveratrol-induced apoptosis may be more effective at lower concentrations if intracellular Hsp72 are depressed. It is worth investigating the effect resveratrol has of surface Hsp72 expression due to the immunogenic properties of surface Hsp72.

In view of this, the aims of the study were:

1. to demonstrate the induction of cell death and apoptosis by resveratrol in a lymphoid (Jurkat) and myeloid (U937) leukaemia cell line

2. to demonstrate that resveratrol sensitizes the cells to TRAIL-induced apoptosis, and to investigate the effect of combination treatment of the cells with resveratrol, ethanol and TRAIL on cell death and apoptosis
3. to investigate the effect of resveratrol on intracellular and surface Hsp72 expression
4. to investigate whether methods to increase or decrease intracellular Hsp72 prior to resveratrol treatment could modulate the sensitivity of the cells to resveratrol-induced cell death and apoptosis

## **2. Methods**

### ***2.1. Reagents and antibodies***

Resveratrol was from Sigma-Aldrich (R5010). A 100mM stock solution of resveratrol was made up in ethanol (99.7%; VWR International, 10107). TRAIL was from PeproTech (310-04) of which a 50mg/ml stock solution was made up in distilled water. Working concentrations of resveratrol, TRAIL and ethanol were made by adding stock solution directly to tissue culture medium.

A 100mM stock solution of Propidium Iodide (PI; Sigma, P4170) was made up in DPBS (Cambrex, BE17-513F) and diluted 1:20 in DPBS to provide a working concentration of 5mM as required. MTS was from Promega (G1112) and made up according to manufacturers' instructions. EnzoLyte Rh110 Caspase-3 assay kit was from Cambridge Bioscience (71141). Hydrogen peroxide (100 volumes solution) was from Fischer (H/1750/15). Trypan Blue (Sigma, T8154) was made up to a 0.4% solution in DPBS.

FITC-conjugated Annexin-V was from BD Bioscience (556 420). FITC-conjugated mouse antihuman Hsp70 monoclonal antibody was from Bioquote (SPA-810-FIH). BD Cytofix/Cytoperm fixation and permeabilization solution was from BD Bioscience (51-2090KZ).

## ***2.2. Cell culture***

Jurkat E6.1 cells and U937 cells were obtained from the European Collection of Cell Culture (Wilshire, UK). Cell lines were maintained in RPMI culture media (Cambrex, BE12-702F) with 10% fetal calf serum (FCS; Cambrex, 14-801F) and 1% antibiotic antimycotic solution (100x; Sigma, A5955). They were kept in a humidified environment with 5% CO<sub>2</sub> at 37°C and maintained at an approximate density of  $5 \times 10^5$  cells/ml by routine passage.

## ***2.3. Treatment of Cells with Compound or Heat Shock***

Prior to any treatments of cells with compound or heat shock, the appropriate cell density for the desired assay was determined. Total cell and viability counts were performed using a haemocytometer on cells diluted 1:1 with Trypan Blue. Preliminary experiments revealed that  $2 \times 10^5$  cells/well was the optimal seeding density for cell viability and caspase-3 assays. A seeding density of  $5 \times 10^4$  cells/well was used for treatments intended for flow cytometry (fluorescence-activated cell sorting; FACS) analysis.

### **2.3.1 Procurement of optimum parameters of heat shock and ethanol treatments for maximum effects on Hsp72 expression**

To determine the heat shock treatment that would sufficiently increase intracellular Hsp72 expression, sufficient volumes of Jurkat and U937 cells were divided into 6-well plates or T25 tissue culture flasks and left in a humidified environment with 5% CO<sub>2</sub> at 42°C (or 37°C for control cells) for 0.5 or 1 hr before being transferred to 37°C for a 0-3 hr recovery period.

To determine the ethanol treatment that would sufficiently decrease intracellular Hsp72 expression, Jurkat and U937 cells were treated with 1.25 % ethanol for various time periods as described in section 2.3.3.

Flow cytometry was used to analyse intracellular Hsp72 after heat shock and ethanol treatments (section 2.5.2).

### **2.3.2. Pre-treatment of cells with heat shock or ethanol**

For pre-treatment of cells with heat shock, cells were kept at 42°C for 1 hr and transferred to 37°C for a 3 hr recovery period before being treated with resveratrol.

For ethanol pre-treatment, Jurkat and U937 cells were treated with 1.25% ethanol in 48-well plates (as described in section 2.3.3) for 2 hours before being centrifuged at

500g for 5 minutes and the supernatant removed. Each cell sample was washed once by re-suspension in 200µL RPMI and centrifuged again at 500g for 5 minutes. Finally, the supernatant was removed and the cell samples were re-suspended in tissue culture medium containing the appropriate concentrations of resveratrol.

Appropriate volumes of resveratrol to ensure that the initial cell density was maintained were used. Cells were treated with resveratrol for 20 hrs as described in section 2.3.3.

### **2.3.3. Treatment of cells with compound**

Jurkat and U937 cells were added to 48-well or 96-well plates at  $5 \times 10^4$  or  $2 \times 10^5$  cells/well (depending on the intended assay) and exposed in triplicate/quadruplicate (for cell viability and caspase-3 assays) or duplicate (for FACS analysis) to various concentrations of treatment compounds. Compounds (resveratrol, TRAIL and ethanol) were used alone or in combination, for the times indicated (e.g. 24 hrs for resveratrol, ethanol and TRAIL treatments alone or in combination, 2 hrs for ethanol pre-treatments, 20 hrs for resveratrol following pre-treatment with ethanol or heat shock, and 0-5 hrs for ethanol alone to determine pre-treatment parameters).

A vector control and dead cell control were included in experiments as appropriate. The vector control comprised of the concentration of ethanol included in the highest concentration of stock resveratrol used. The dead cell control included for cell viability



and caspase-3 assays was achieved by adding a 1:1 volume ratio of hydrogen peroxide to the assigned cell samples. For FACS analysis, heat shocked cells were included as dead cell controls after incubating them in a water bath at 42°C for 2 hrs.

## ***2.4. Microtiter plate based assays***

### **2.4.1. Cell viability – MTS assay**

Treated cells were centrifuged at 500g for 5 minutes in microcentrifuge tubes and the supernatant discarded. The cells were resuspended in 100µL tissue culture media and transferred to a 96-well plate. 20µl of MTS solution was mixed into each cell suspension and the plate was incubated in the dark at 37°C for 1.5 – 2 hours. Absorbance of the formazan product was measured at 490 nm on a bi-spectral fluorescent spectrophotometer (Bio-Tek).

### **2.4.2. Cell viability – Propidium Iodide (PI) Assay**

Treated cells were centrifuged at 500g for 5 minutes in microcentrifuge tubes and the supernatant discarded. The cells were resuspended in 50µl tissue culture media and transferred to a 96-well plate. 20µl working concentration of PI was mixed into each cell suspension and the plate was incubated in the dark at room temperature for 20

minutes. Fluorescence was measured at Ex/Em 535/617 on a bi-spectral fluorescent spectrophotometer (Bio-Tek).

#### **2.4.3. Apoptosis – Caspase-3 assay**

Treated cells were centrifuged at 500g for 5 minutes in microcentrifuge tubes and the supernatant discarded. The cells were resuspended in 150µl tissue culture media and transferred to a 96-well plate. 50µl caspase-3 substrate solution (Cambridge Bioscience), made according to the manufacturers instructions, was mixed in to each cell suspension after which the cells were incubated in the dark at room temperature for 1 hour. Fluorescence was measured at Ex/Em 495/530 on a bi-spectral fluorescent spectrophotometer (Bio-Tek).

### ***2.5. Flow cytometry***

#### **2.5.1. Annexin V and PI analysis**

Treated cells were centrifuged at 500g for 5 minutes in microcentrifuge tubes and the supernatant discarded. The cells were washed once by resuspending them in 150µl wash buffer (5% FCS in DPBS) and then transferred to a 96-well V-bottomed plate in which they were centrifuged at 500g for 5 minutes and the supernatant removed.

FITC-conjugated annexin V and PI were diluted 1:20 and 1:40, respectively, in binding buffer (0.1M HEPES/NaOH, 1.4M NaCl, 25 mM CaCl<sub>2</sub>). 50µl of the diluted FITC-conjugated annexin V was added to each well and incubated in the dark at room temperature for 20 minutes, after which 100µl of the diluted PI was added to each well and the cells analysed using the BD FACS CANTO Dual Laser Flow Cytometer (Becton Dickinson). Control cell suspensions with no stain, only annexin V or only PI staining as well as heat shocked dead cell controls were included in the analysis. Equal volume of binding buffer was added to the wells where annexin V or PI was omitted.

### **2.5.2. Analysis of intracellular and surface Hsp70**

Treated cells were centrifuged at 500g for 5 minutes in microcentrifuge tubes and the supernatant discarded. The cells were washed once by resuspending them in 100µl wash buffer (5% FCS in DPBS) and then transferred to a 96-well V-bottomed plate in which they were centrifuged at 500g for 5 minutes and the supernatant removed.

For intracellular Hsp70 analysis, the cells were permeabilized prior to incubation with antibody by adding 70µl permeabilizing solution to each sample and incubating for 20 minutes in the dark at room temperature. The cells were washed by re-suspension in 100µl wash buffer and centrifugation at 500g for 5 minutes, after which the supernatant was discarded. This step was omitted for surface Hsp70 analysis where anti-Hsp70 was added directly after the first wash.

FITC-conjugated anti-Hsp70 (human) was diluted 1:2500 in wash buffer and 50µl of the mixture was added to each well except wells containing untreated 'no stain' control cells. The cells were incubated for 40 minutes in the dark at room temperature after which 100µl wash buffer was added to each well and the plate centrifuged at 500g for 5 minutes and the supernatant discarded. All samples were resuspended in 150µl DPBS and analysed using the BD FACS CANTO Dual Laser flow cytometer (Becton Dickinson).

## ***2.6. Statistical Analysis***

GraphPad Prism 5.0 was used for statistical analysis and graphical representation of results. Results were presented as mean  $\pm$  standard error of mean (SEM). Statistical significance of differences in means was conducted when triplicate or quadruplicate results were available, and analysed using one-way ANOVA with post-hoc Dunnett or Bonferoni analysis or two-tailed unpaired Student's *t*-test. When results were available in duplicate only, non-linear regression was used to analyse the fit of the data onto a curve.

### 3. Results

#### *3.1. Cell viability and apoptosis assays explained*

The PI and MTS assays are two methods to measure cell viability. Whilst PI fluorescence in the PI assay indicates loss of membrane integrity, formazan product development in the MTS assay is a measure of mitochondrial activity. PI is a fluorescent molecule that binds to DNA, but is unable to permeate intact cell membranes and can therefore be used to differentiate viable and dead cells. Loss of membrane integrity is a feature of necrotic and late apoptotic cells and therefore these cells allow PI binding, whilst viable cells do not. The MTS assay utilises the mechanism by which mitochondrial enzyme activity is required for the reduction of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and phenazine methosulfate to form a purple formazan product. The formazan product has an absorbance maximum at 490nm, and the level of absorbance is directly proportional to the mitochondrial activity of the cell sample. Loss of mitochondrial activity is an unspecific feature of loss of viability in different types of cell death e.g. apoptosis and necrosis.

The EnzoLyte Rh110 Caspase-3 Assay is a specific measure of apoptosis since activated caspase-3 is a key player in the apoptotic pathway. It measures caspase-3 activity indirectly by measurement of the fluorescence of the Rh110 fluorophore

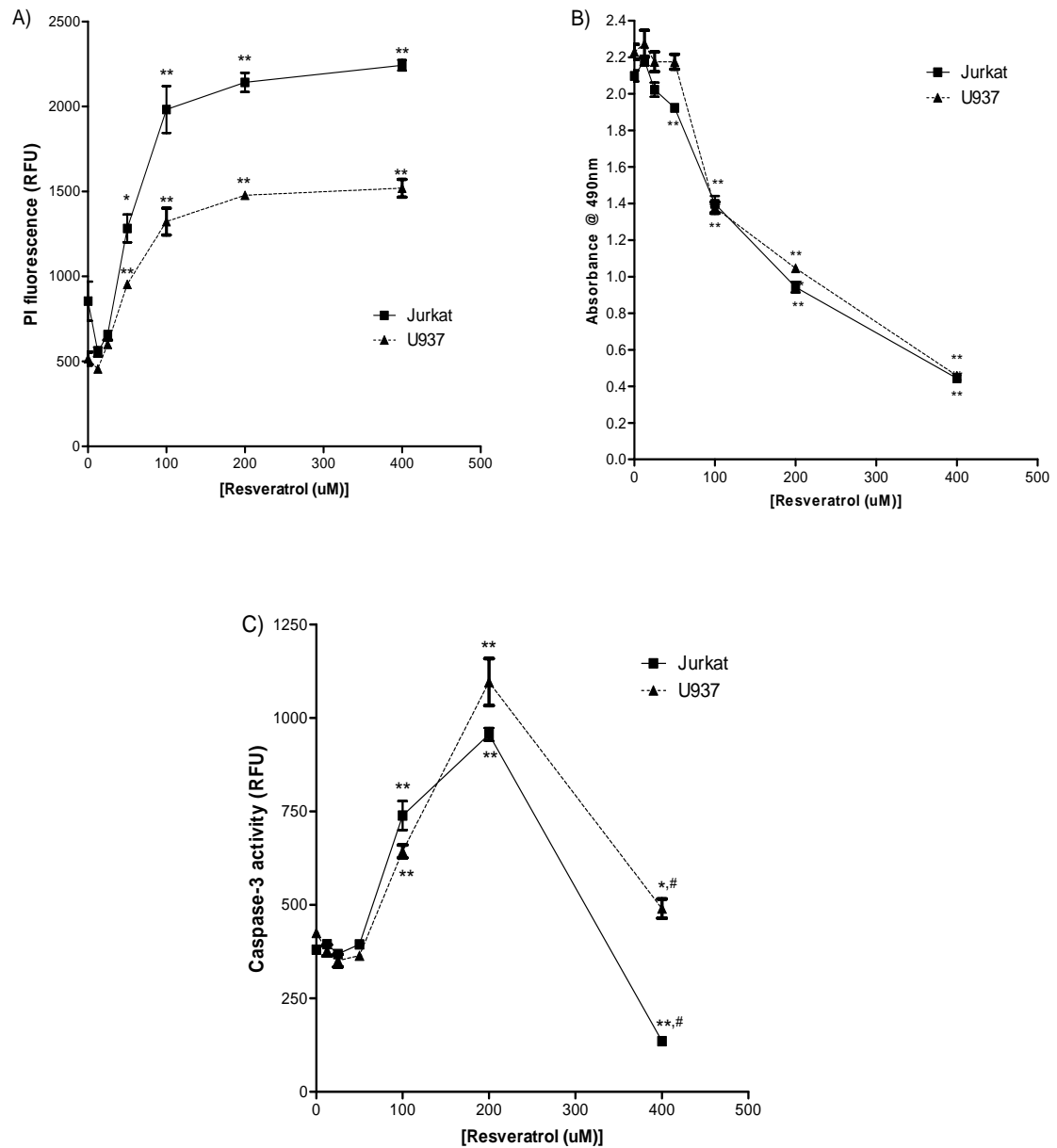
produced by the cleavage of (Z-DEVD)<sub>2</sub>-Rh110 by active caspase-3 (AnaSpec product information).

Annexin V binding is also a specific measure of apoptosis, particularly early apoptosis. One of the first appearing features of apoptosis is the translocation of phosphatidylserine (PS) from the inner cell membrane to the cell surface. Annexin V has a high affinity for PS and thus, its binding and fluorescence of the conjugated FITC molecule indicates exposure of PS to the extracellular environment.

### ***3.2. Resveratrol causes cell death in a dose-dependent manner***

Initial experiments demonstrated that resveratrol causes cell death in a dose dependent manner in Jurkat and U937 cells, corresponding to caspase-3 dependent apoptosis at 100-200 $\mu$ M and necrosis at higher concentrations of resveratrol (see figure 3.1).

The PI assay revealed dose-dependent loss of membrane integrity reaching statistical significance at 50 $\mu$ M and above for both cell lines ( $p < 0.05$ , figure 3.1A). Similarly, the MTS assay revealed dose-dependent loss of mitochondrial activity with statistical significance at 50 $\mu$ M and above in Jurkat cells ( $p < 0.01$ ) and 100 $\mu$ M and above in U937 cells ( $p < 0.01$ , figure 3.1B). Loss of membrane integrity and mitochondrial activity independently indicate late apoptosis and necrosis. Thus, the PI and MTS assays demonstrated similar effects of resveratrol on cell viability. For simplicity, later cell viability experiments were therefore based on the PI assay alone.



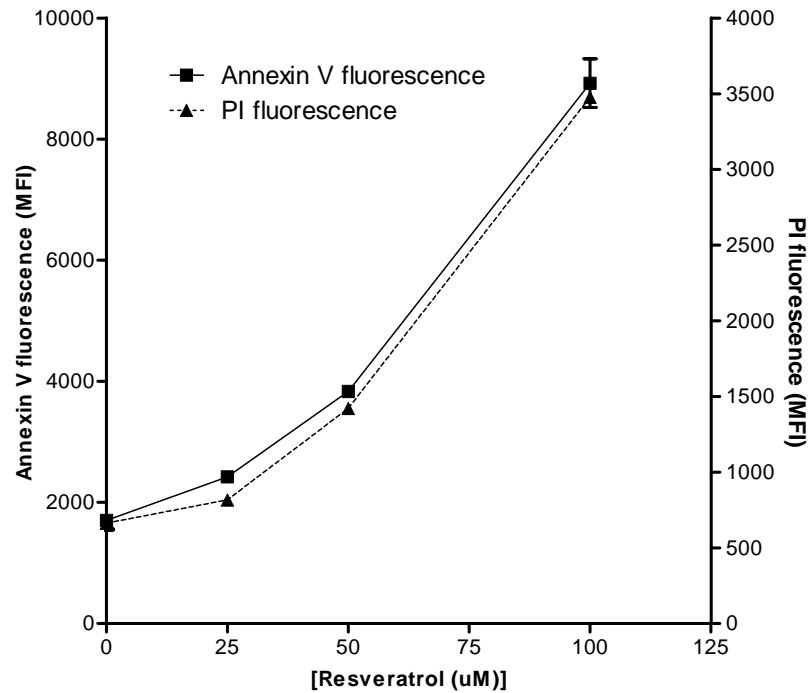
**Figure 3. 1. Dose-dependent induction of cell death via apoptosis and necrosis by resveratrol.**

A) PI fluorescence, B) formazan absorbance, and C) caspase-3 activity of Jurkat and U937 cells treated with various concentrations of resveratrol for 24 hours and analysed using the PI, MTS or caspase-3 assay. Cell viability as measured by PI fluorescence was significantly reduced by 50–400 $\mu$ M resveratrol in both cell lines (A;  $p < 0.05$  or  $p < 0.01$ ). Cell viability as measured by formazan absorbance was significantly reduced by 50–400 $\mu$ M resveratrol in U937 cells (B;  $p < 0.05$  or  $p < 0.01$ ) and 100–400 $\mu$ M in Jurkat cells (B;  $p < 0.01$ ). Caspase-3 activity was significantly increased by 100–200 $\mu$ M resveratrol (C;  $p < 0.01$ ) and decreased at 400 $\mu$ M compared with 200 $\mu$ M in both cell lines (C;  $p < 0.001$ ). Values are shown as mean  $\pm$  standard error (SEM) of samples in quadruplicate. Statistical significance was analysed using one-way ANOVA with post-hoc Dunnett test and two-tailed unpaired Student's  $t$ -tests. Treated versus untreated cells: \*  $p < 0.05$ , \*\*  $p < 0.01$ . Cells treated with 200 $\mu$ M versus 400 $\mu$ M resveratrol: #  $p < 0.001$ .

The caspase-3 assay showed that 100-200 $\mu$ M resveratrol caused increased caspase-3 activity ( $p<0.01$ ), and caspase-3 activity was significantly reduced at the higher concentration of 400 $\mu$ M ( $p<0.01$  in Jurkat cells and  $p<0.05$  in U937 cells; see figure 3.1C), indicating a shift from apoptosis to necrosis as the main form of cell death above 200 $\mu$ M resveratrol. A raise in caspase-3 activity was not seen at concentrations of resveratrol  $<100\mu$ M despite such concentrations causing significant reduction in cell viability.

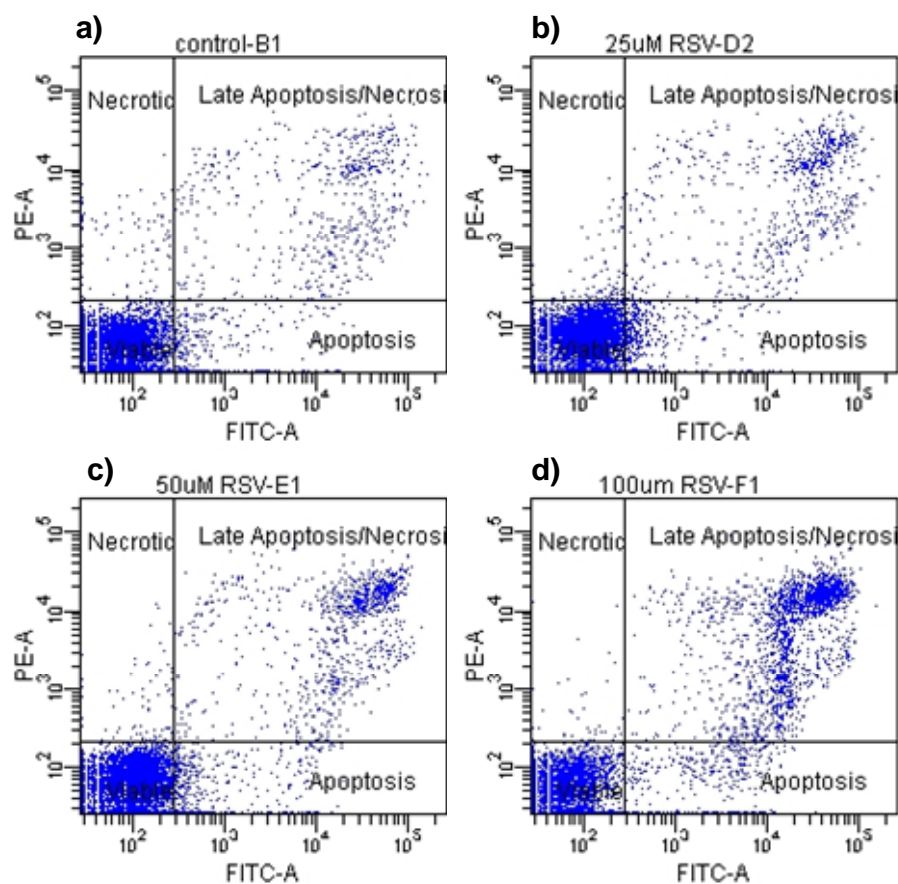
The above findings were confirmed by measuring annexin V binding and PI fluorescence of resveratrol-treated cells using flow cytometry. Dose-dependent loss of cell viability as measured by PI fluorescence corresponded to an increase in annexin V presentation at 25, 50 and 100 $\mu$ M resveratrol (see figure 3.2). Flow cytometry dot plots revealed that the majority of dying cells at all concentrations caused both annexin V binding and PI fluorescence, suggesting late apoptosis/necrosis (see figure 3.3). Resveratrol caused a reduction in cell viability from 91% viable cells in the untreated sample to 63% viable cells in the sample treated with 100 $\mu$ M resveratrol (see figure 3.4).





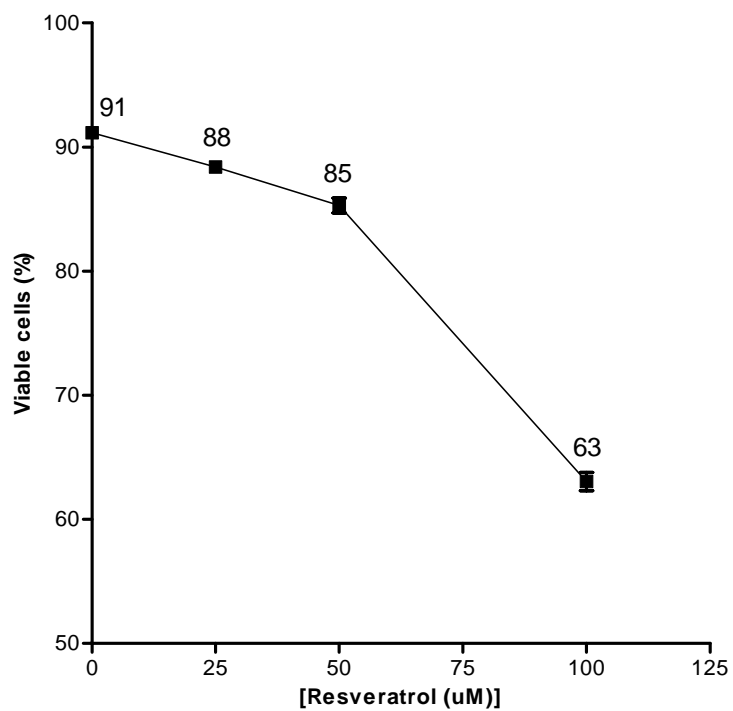
**Figure 3. 2. Dose-dependent induction of cell death by resveratrol in U937 cells, as analysed with flow cytometry.**

U937 cells treated with increasing concentrations of resveratrol for 24 hours caused a dose-dependent increase in Annexin V presentation as measured by fluorescence of FITC-conjugated Annexin V, and a dose-dependent loss of cell viability as measured by PI fluorescence. Statistical analysis was limited due to there being only two replicates per sample. Non-linear regression was performed, and  $R^2 > 0.99$  against a quadratic curve fit for both data sets. This suggests that both curves are quadratic in nature and that there are statistically significant increases in FITC-Annexin V and PI fluorescence. Values are shown as mean  $\pm$  SEM of samples performed in duplicate.



**Figure 3.3. Dot plots from flow cytometry analysis of resveratrol (RSV)-treated cells showing distribution of the cell population based on state of viability.**

U937 cells were treated with 0 $\mu$ M (a), 25 $\mu$ M (b), 50 $\mu$ M (c) and 100 $\mu$ M (d) resveratrol for 24 hours and apoptosis and cell viability determined using the Annexin V and PI assays using flow cytometry. PE-A indicates PI fluorescence (mean fluorescence intensity; MFI) and FITC-A indicates fluorescence of FITC-conjugated Annexin V (MFI). The bottom left quadrant of the plots shows the viable cell population that did not cause either PI or FITC-Annexin V fluorescence; the top left quadrant shows the cell population that displayed necrosis by impaired membrane integrity and PI fluorescence only; the bottom right quadrant shows the cell population displaying apoptosis by Annexin V binding and FITC fluorescence only; and the top right quadrant shows the cell population displaying late apoptosis/necrosis by causing both PI and FITC fluorescence, thus indicating apoptosis by allowing Annexin V binding and necrosis by the impaired membrane integrity. The dot plots reveal that the unviable cell populations were mostly in late apoptotic/necrotic state and the unviable population size increased with increasing concentrations of resveratrol.

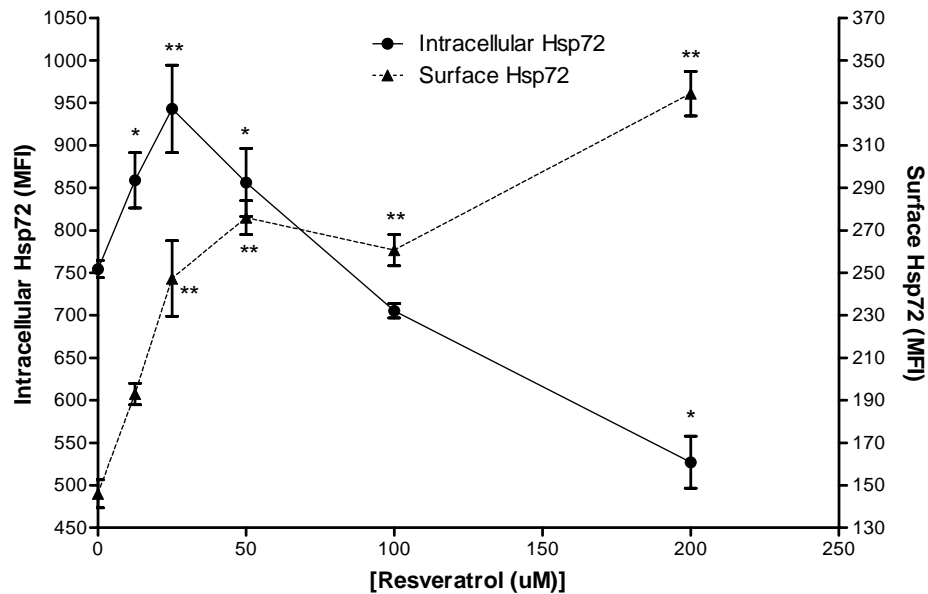


**Figure 3. 4. *Resveratrol causes a dose-dependent reduction in cell viability as measured by flow cytometry.***

Flow cytometry analysis of U937 cells treated with 0-100 $\mu$ M resveratrol indicated progressive loss of cell viability measured by the fluorescence of either FITC-conjugated Annexin V or PI. Values are shown as mean  $\pm$  SEM of samples performed in duplicate.

***3.3. Low concentrations of resveratrol cause raised intracellular and surface levels of Hsp72; higher concentrations of resveratrol cause further increase in surface levels of Hsp72 but no change or decrease in intracellular levels of Hsp72***

Intracellular and surface levels of Hsp72 of Jurkat cells treated with resveratrol for 24 hours were analysed using flow cytometry. Cells treated with 12.5, 25 and 50 $\mu$ M resveratrol had significantly higher levels of intracellular Hsp72 than untreated cells ( $p<0.05$ ; see figure 3.5). In cells treated with 100 $\mu$ M, the levels were unchanged from those of untreated cells, and above 100 $\mu$ M the levels were significantly reduced, presumably due to the higher degree of necrosis occurring at these higher concentrations. Surface levels of Hsp72 increased with increasing concentrations of resveratrol, statistically significant at 25 $\mu$ M and above ( $p<0.01$ ; see figure 3.5). The results indicate that lower concentrations of resveratrol (12.5-50 $\mu$ M) induces increased Hsp72 expression at a sufficient level to allow increased intracellular levels as well as export for elevated surface expression. It is assumed that the increased degree of necrosis occurring at higher concentrations of resveratrol is responsible for the appearance of increased surface expression of Hsp72, and that this is not a true reflection of changes in cellular Hsp72 expression.



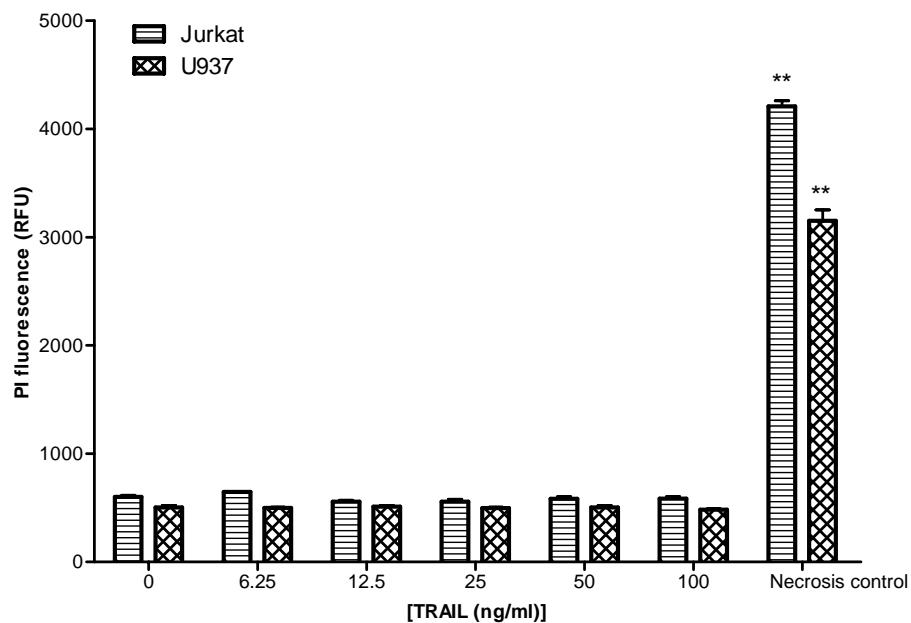
**Figure 3. 5. Intracellular and surface Hsp72 of Jurkat cells treated with resveratrol.**

Jurkat cells treated with various concentrations of resveratrol for 24 hours were analysed for levels of intracellular and surface Hsp72 by anti-Hsp72 binding using flow cytometry. Levels of intracellular Hsp72 were significantly increased by 12.5, 25 and 50μM resveratrol ( $p < 0.05$  or  $p < 0.01$ ), unchanged by 100 μM, and decreased from control by 200μM resveratrol ( $p < 0.05$ ). Levels of surface Hsp72 were significantly increased by treatment with 25, 50, 100 and 200μM resveratrol ( $p < 0.01$ ). Values are shown as mean  $\pm$  SEM of samples done in triplicate. Statistical significance was analysed using one-way ANOVA and post-hoc Dunnett test. Treated versus untreated cells: \*  $p < 0.05$ , \*\*  $p < 0.01$ .

### ***3.4. Resveratrol sensitizes leukaemia cells to TRAIL-induced apoptosis and displays a synergistic effect with ethanol on cell death and TRAIL-induced apoptosis***

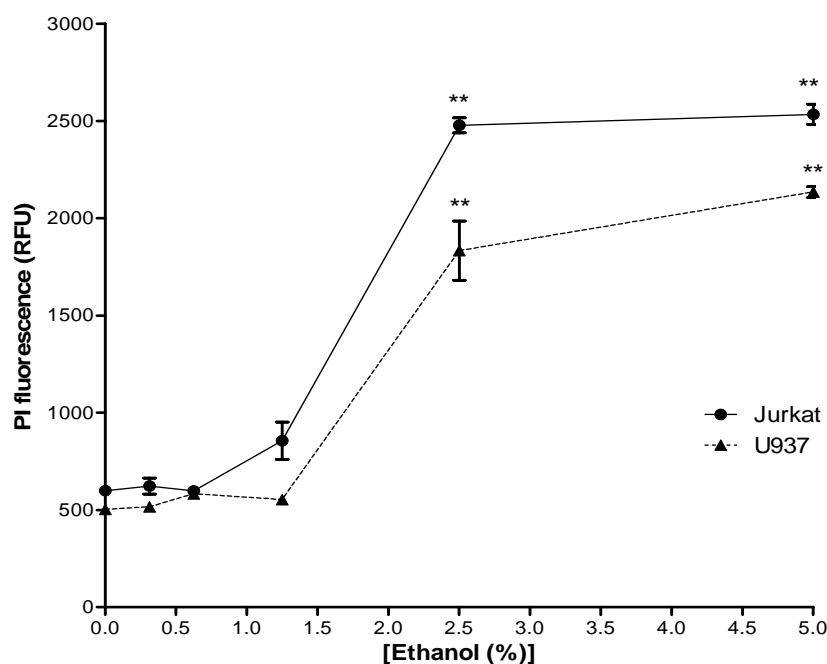
Dose response experiments with TRAIL and ethanol were performed on Jurkat and U937 cells using the PI assay to determine sub-lethal concentrations of a 24 hr treatment. No concentration of TRAIL (0-100ng/ml) had any effect on cell viability (figure 3.6), indicating that higher concentrations are required to cause cell death in the

cell lines used. Concentrations of ethanol above 1.25% significantly reduced cell viability in both cell lines ( $p < 0.01$ ; figure 3.7), whilst 1.25% and lower concentrations remained sub-lethal. 25ng/ml TRAIL and 1.25% ethanol have been successfully used as sub-lethal concentrations on Jurkat cells in the laboratory previously. Therefore, these concentrations were initially selected for use in combination treatment experiments.



**Figure 3. 6. Effect on cell viability of various concentrations of TRAIL.**

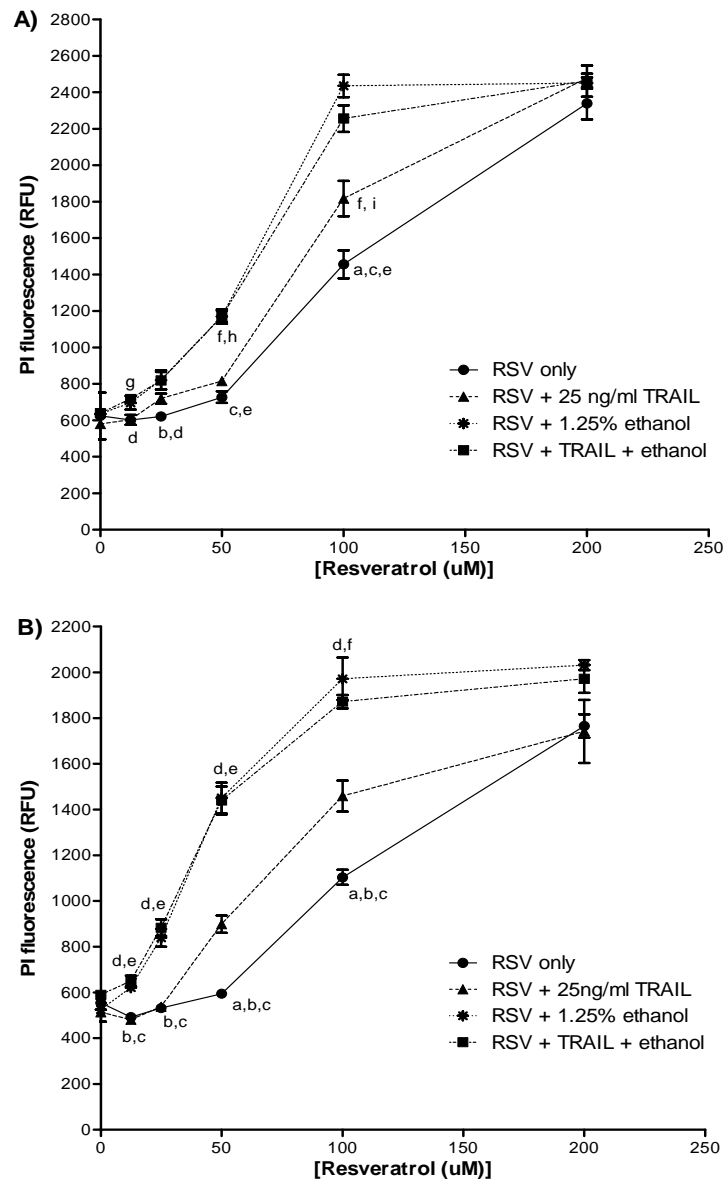
Jurkat (striped fill) and U937 (crossed fill) cells were treated with various concentrations of TRAIL for 24 hours and cell viability analysed with the PI assay. Cells treated with hydrogen peroxide acted as necrosis controls. Only the necrosis controls showed a statistically significant reduction in cell viability compared with untreated cells. Values are shown as mean  $\pm$  SEM of samples performed in quadruplicate. Statistical significance was analysed using one-way ANOVA and post-hoc Dunnett test. Treated versus untreated cells: \*\*  $p < 0.01$ .



**Figure 3. 7. Effect of ethanol treatment on cell viability.**

Jurkat (solid line) and U937 (dashed line) cells were treated with various concentrations of ethanol for 24 hours and cell viability analysed with the PI assay. Statistically significant reduction in cell viability compared with untreated cells was seen at 2.5% and 5% ethanol ( $p < 0.01$ ). Values are shown as mean  $\pm$  SEM of samples performed in quadruplicate. Statistical significance was analysed using one-way ANOVA and post-hoc Dunnett test. Treated versus untreated cells: \*\*  $p < 0.01$ .

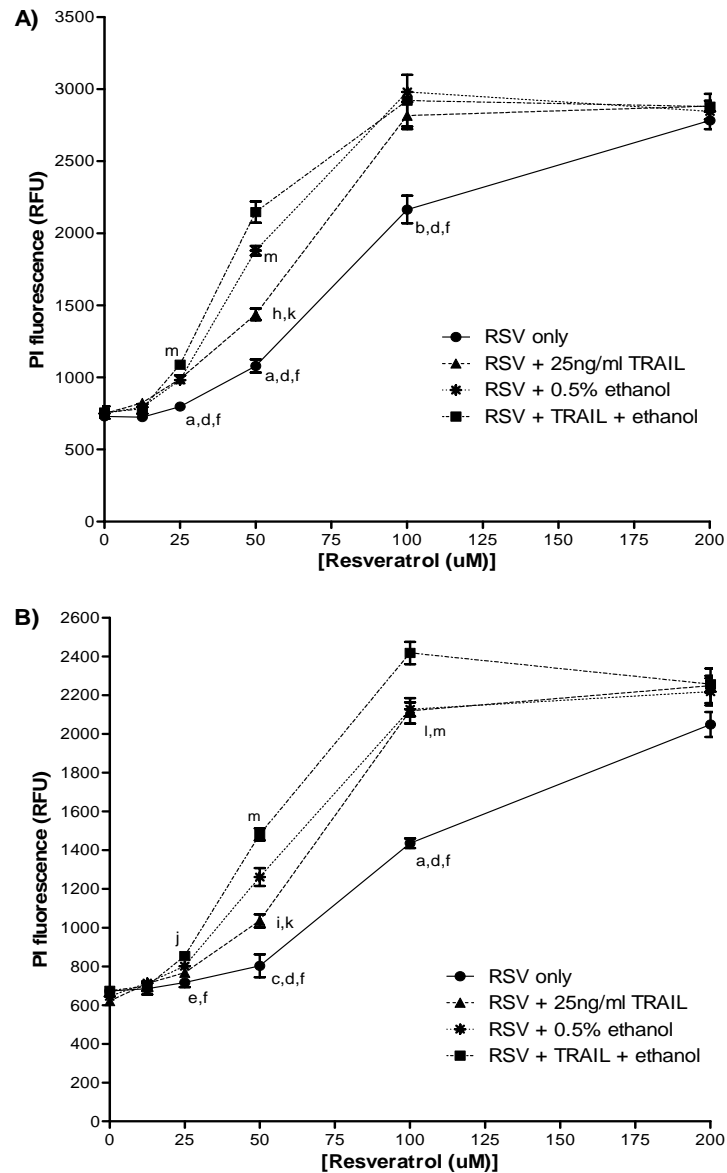
Dose response experiments with resveratrol were performed on Jurkat and U937 cells treated alone or alongside 25ng/ml TRAIL, 1.25% ethanol or both. Cell viability was analysed using the PI assay. TRAIL and ethanol both had independently additive effects to resveratrol on loss of cell viability ( $p < 0.05$ ; figure 3.8). However, the combination of all three compounds produced no greater effect than the combination of resveratrol and 1.25% ethanol. It was thought that the large effect on cell viability caused by resveratrol in combination with 1.25% ethanol masked a possible extra additive effect of TRAIL. Therefore, the ethanol concentration was reduced to 0.5% and the experiment repeated.



**Figure 3. 8. Effect on cell viability of combination treatments with resveratrol (RSV) and 25ng/ml TRAIL, or 1.25% ethanol, or both.**

Jurkat (A) and U937 (B) cells were treated with various concentrations of resveratrol alone or together with 25ng/ml TRAIL or 1.25% ethanol or both for 24 hours and cell viability analysed with the PI assay. TRAIL and ethanol appeared to both have independently additive effects on the dose-dependent loss of cell viability induced by resveratrol, but no additive effect to resveratrol of TRAIL and ethanol in combination with each other was seen. Values are shown as mean  $\pm$  SEM of samples performed in quadruplicate. Statistical significance was analysed using one-way ANOVA and post-hoc Bonferoni test. Statistical significance: a)  $p < 0.05$  between 'RSV only' and 'RSV + TRAIL'; b)  $p < 0.01$  between 'RSV only' and 'RSV + TRAIL'; c)  $p < 0.05$  between 'RSV only' and 'RSV + ethanol'; d)  $p < 0.001$  between 'RSV only' and 'RSV + ethanol'; e)  $p < 0.05$  between 'RSV only' and 'RSV + TRAIL + ethanol'; f)  $p < 0.001$  between 'RSV only' and 'RSV + TRAIL + ethanol'; g)  $p < 0.001$  between 'RSV + TRAIL' and 'RSV + ethanol'; h)  $p < 0.05$  between 'RSV + TRAIL' and 'RSV + TRAIL + ethanol'; i)  $p < 0.001$  between 'RSV + TRAIL' and 'RSV + TRAIL + ethanol'; j)  $p < 0.01$  between 'RSV + TRAIL' and 'RSV + TRAIL + ethanol'.

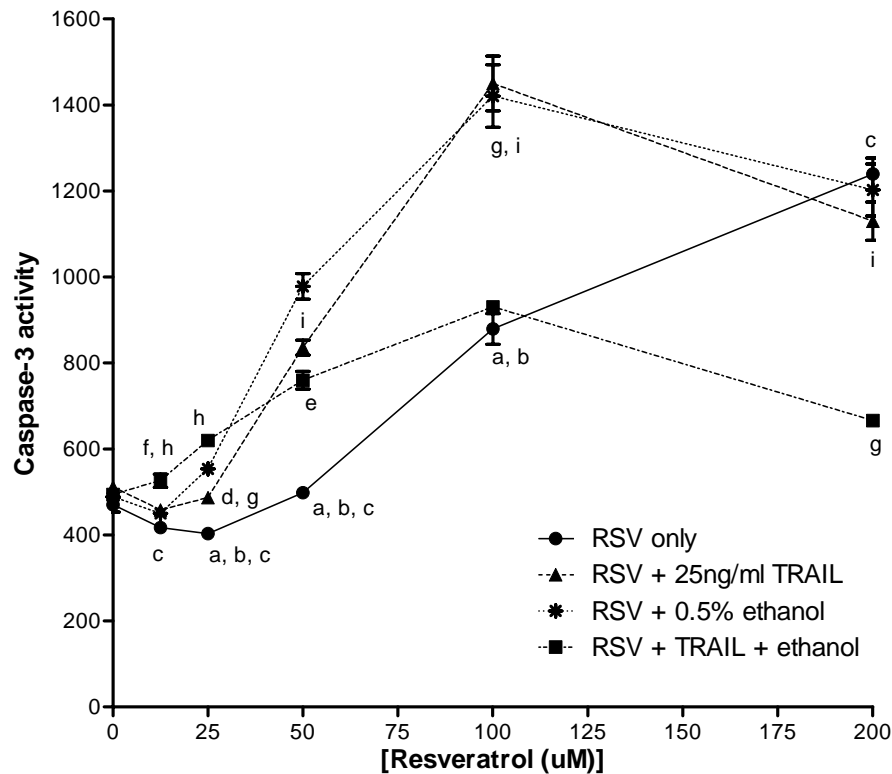




**Figure 3. 9. Effect on cell viability of combination treatments with resveratrol (RSV) and 25ng/ml TRAIL, or 0.5% ethanol, or both.**

Jurkat (A) and U937 (B) cells were treated with various concentrations of resveratrol alone or together with 25ng/ml TRAIL or 0.5% ethanol or both for 24 hours and cell viability analysed with the PI assay. TRAIL and ethanol appeared to both have independently additive effects as well as a combined additive effect on the dose-dependent loss of cell viability induced by resveratrol. Values are shown as mean  $\pm$  SEM of samples done in quadruplicate. Statistical significance was analysed using one-way ANOVA and post-hoc Bonferoni test. Statistical significance: a)  $p < 0.001$  between 'RSV only' and 'RSV + TRAIL'; b)  $p < 0.01$  between 'RSV only' and 'RSV + TRAIL'; c)  $p < 0.05$  between 'RSV only' and 'RSV + TRAIL'; d)  $p < 0.001$  between 'RSV only' and 'RSV + ethanol'; e)  $p < 0.01$  between 'RSV only' and 'RSV + ethanol'; f)  $p < 0.001$  between 'RSV only' and 'RSV + TRAIL + ethanol'; g)  $p < 0.01$  between 'RSV only' and 'RSV + TRAIL + ethanol'; h)  $p < 0.001$  between 'RSV + TRAIL' and 'RSV + ethanol'; i)  $p < 0.05$  between 'RSV + TRAIL' and 'RSV + ethanol'; j)  $p < 0.01$  between 'RSV + TRAIL' and 'RSV + TRAIL + ethanol'; k)  $p < 0.001$  between 'RSV + TRAIL' and 'RSV + TRAIL + ethanol'; l)  $p < 0.05$  between 'RSV + TRAIL' and 'RSV + TRAIL + ethanol'; m)  $p < 0.05$  between 'RSV + ethanol' and 'RSV + TRAIL + ethanol'.

Cell viability was again analysed using the PI assay and apoptosis using the caspase-3 assay. With 0.5% ethanol, additive effects on cell viability and apoptosis/necrosis were seen with all combination treatments and the largest effect seen with the triple combination of resveratrol, 0.5% ethanol and 25ng/ml TRAIL ( $p<0.05$ ; figures 3.9 and 3.10). Double treatments with resveratrol and 0.5% ethanol or resveratrol and 25ng/ml TRAIL had similar effects on cell death and apoptosis, although resveratrol and 0.5% ethanol was somewhat more apoptotic/cytotoxic causing significantly greater cell death at 50 $\mu$ M resveratrol and significantly greater caspase-3 activity at 25 and 50 $\mu$ M resveratrol ( $p<0.05$ ). The additive effect of triple treatment was particularly prominent with 50 $\mu$ M resveratrol in the PI assay ( $p<0.05$ ), and 25 $\mu$ M resveratrol in the caspase-3 assay ( $p<0.01$ ). Whereas no decrease of caspase-3 activity was seen with single treatment with resveratrol, caspase-3 activity decreased at concentrations above 100 $\mu$ M in the double treatments, and above 50 $\mu$ M in the triple combination treatments. This indicates a shift from apoptosis to necrosis occurring at lower concentrations of resveratrol, and thus greater levels of toxicity occurring with combination treatments.



**Figure 3. 10. Effect of combination treatments with resveratrol (RSV) and 25ng/ml TRAIL, or 0.5% ethanol, or both, on caspase-3 dependent apoptosis.**

U937 cells were treated with various concentrations of resveratrol alone or together with 25ng/ml TRAIL or 0.5% ethanol or both for 24 hours and apoptosis measured with the caspase-3 assay. TRAIL and ethanol appeared to both have independently additive effects as well as a combined additive effect on the dose-dependent apoptotic-to-necrotic effect of resveratrol. A significant increase in caspase-3 activity was measured with each treatment added to resveratrol at 25μM ( $p<0.001$ ). The highest caspase-3 activity achieved after single treatment was with 200μM resveratrol, whereas cells treated with 200μM + TRAIL, ethanol or both displayed decreased apoptosis from a peak at 100μM resveratrol, suggesting a shift to necrosis. Values are shown as mean  $\pm$  SEM of samples done in quadruplicate. Statistical significance was analysed using one-way ANOVA and post-hoc Bonferoni test. Statistical significance:

- a)  $p<0.001$  between 'RSV only' and 'RSV + TRAIL'
- b)  $p<0.001$  between 'RSV only' and 'RSV + ethanol'
- c)  $p<0.001$  between 'RSV only' and 'RSV + TRAIL + ethanol'
- d)  $p<0.01$  between 'RSV + TRAIL' and 'RSV + ethanol'
- e)  $p<0.05$  between 'RSV + TRAIL' and 'RSV + ethanol'
- f)  $p<0.01$  between 'RSV + TRAIL' and 'RSV + TRAIL + ethanol'
- g)  $p<0.001$  between 'RSV + TRAIL' and 'RSV + TRAIL + ethanol'
- h)  $p<0.01$  between 'RSV + ethanol' and 'RSV + TRAIL + ethanol'
- i)  $p<0.001$  between 'RSV + ethanol' and 'RSV + TRAIL + ethanol'

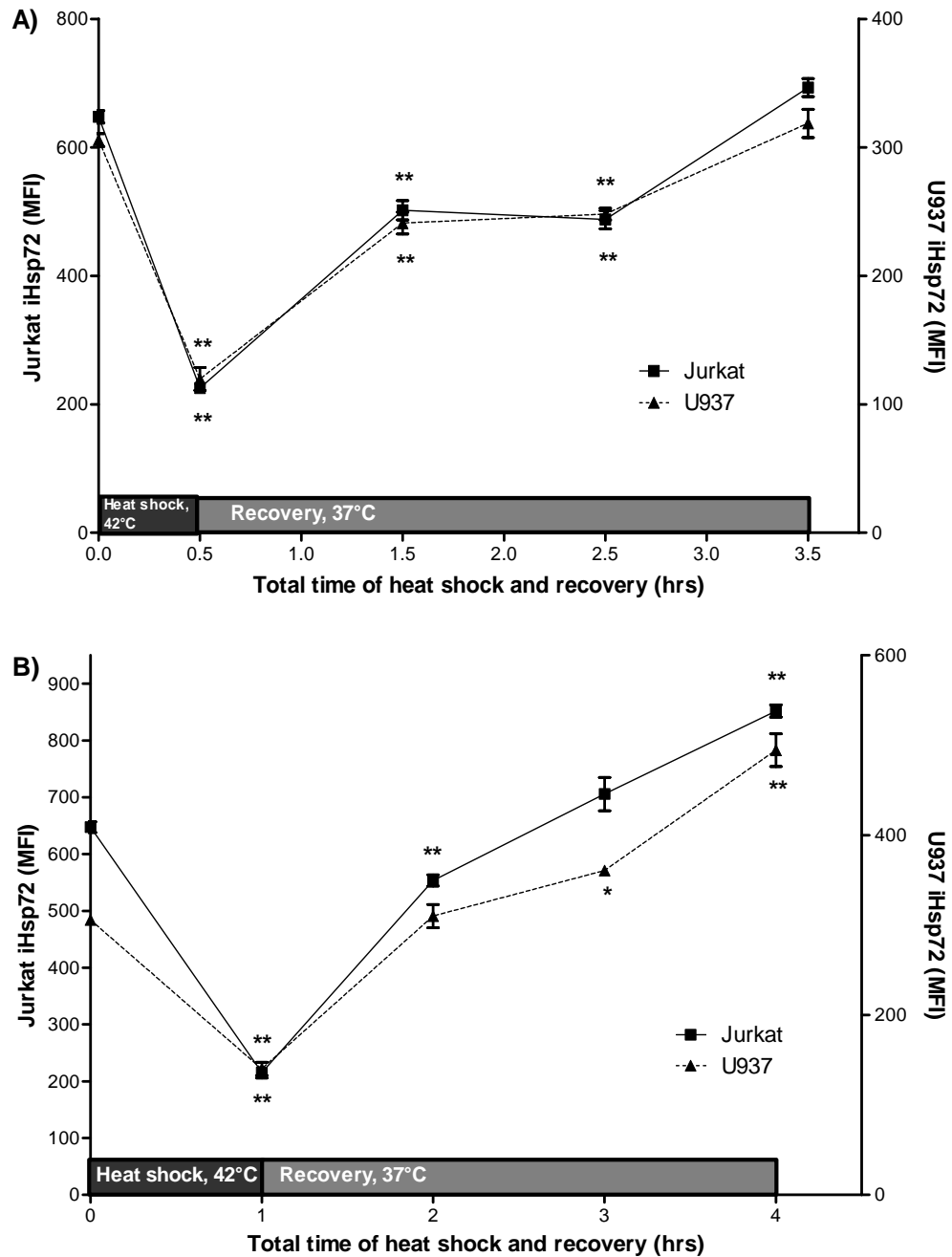
The results were confirmed by microscope views of treated cells showing greater degrees of apoptosis and necrosis at higher concentrations of compounds used. The additive effect of TRAIL on the effect of resveratrol on cell death was also confirmed by cell counts of samples treated using the trypan blue exclusion test (data not shown).

### ***3.5. Manipulation of Hsp72 expression prior to resveratrol treatment***

To determine whether modification of intracellular Hsp72 levels can alter sensitivity of cells to resveratrol-induced cell death, Jurkat and U937 cells were exposed to mild, non-lethal heat shock or 1.25% ethanol prior to treatment with resveratrol for 20 hrs.

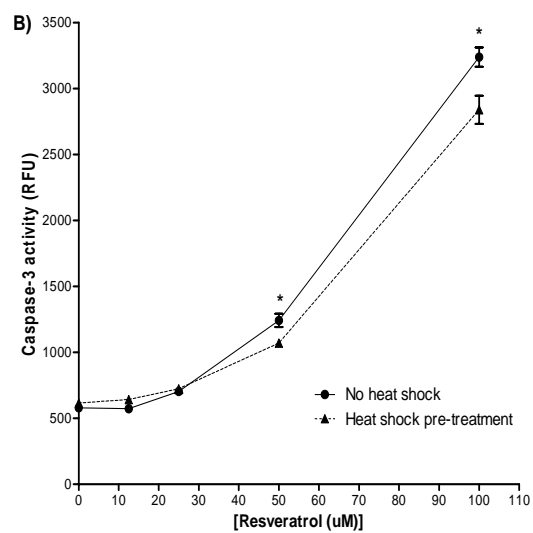
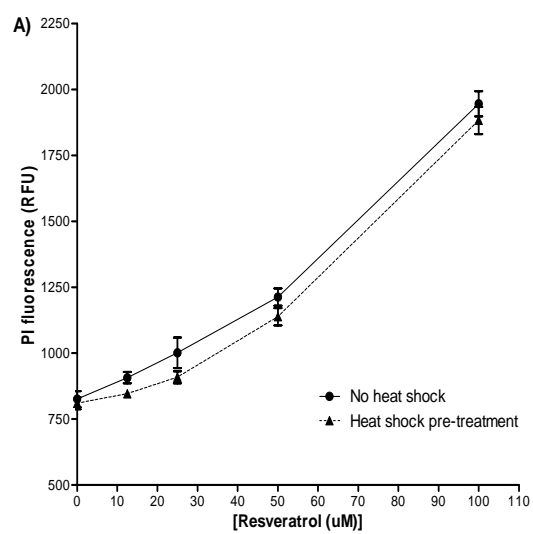
#### **3.5.1. Stimulating Hsp72 expression by pre-treating cells to heat shock prior to resveratrol treatment increases resistance of cells to resveratrol-induced cell death**

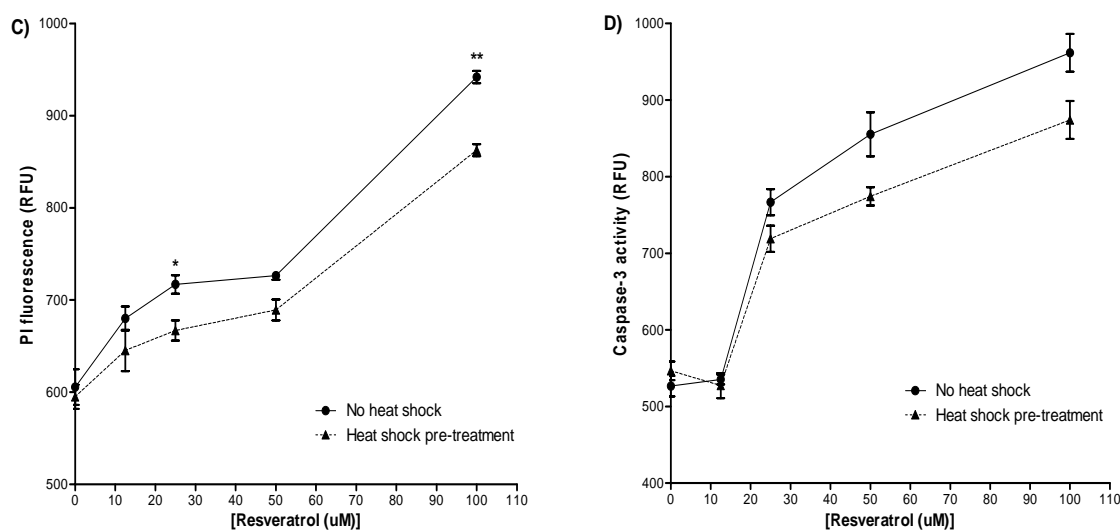
Jurkat and U937 cells were subjected to various time periods of heat shock and recovery in order to determine parameters to induce Hsp72 expression without causing cell death. Initial experiments revealed that a 2 hr heat shock at 42°C caused significant cell death (data not shown), whereas 1 hr was known not to cause cell death (by communication with Nina Dempsey). Therefore 0.5 and 1 hr heat shock incubations were used. The greatest level of intracellular Hsp72 was obtained after an 1 hr incubation at 42°C followed by a 3 hr recovery period at 37°C ( $p<0.01$ ; figure 3.11).



**Figure 3. 11. The effect of mild heat shock on levels of intracellular Hsp72 (iHsp72).**

Jurkat and U937 cells were subjected to heat shock for 0.5 hour (A) or 1 hour (B) by incubation at 42°C, then allowed to recover at 37°C for 0–3 hours before iHsp72 levels by anti-Hsp72 binding was analysed using flow cytometry. Levels of iHsp72 were significantly decreased at 0-2 hours recovery following 0.5 hour heat shock ( $p < 0.01$ ), and not significantly increased from untreated cells after 3 hours recovery. Levels of iHsp72 were significantly decreased at 0-1 hour recovery in Jurkat cells ( $p < 0.05$ ), and 0 and 2 hours recovery in U937 cells ( $p < 0.05$  and  $p < 0.01$ ), but significantly increased after 3 hours recovery in both cell lines ( $p < 0.01$ ). Values are shown as mean  $\pm$  SEM of samples conducted in triplicate for U937 cells and quadruplicate for Jurkat cells. Statistical significance was analysed using one-way ANOVA and post-hoc Dunnett test. Treated cells versus untreated cells: \*  $p < 0.05$ , \*\*  $p < 0.01$ .





**Figure 3. 12. Pre-treatment of cells with heat shock results in increased resistance to resveratrol-induced cell death.**

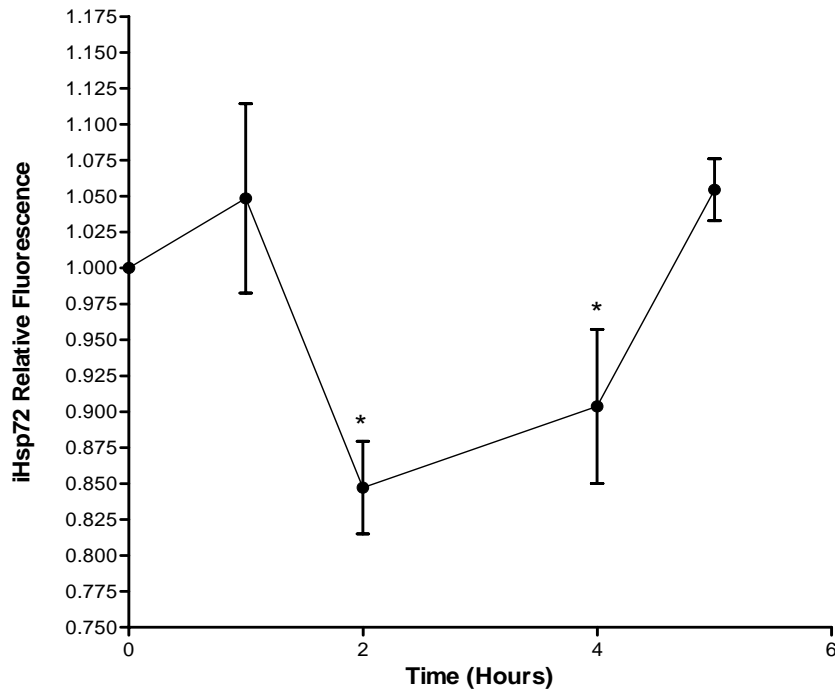
Jurkat (A, B) and U937 (C, D) cells were incubated with various concentrations of resveratrol for 20 hours following an initial expose to heat shock (1 hour incubation at 42°C followed by a 3 hours recovery period at 37°C) or no heat shock (control cells were kept at 37°C for the initial 4 hours). After treatment, a PI assay (A, C) and caspase-3 assay (B, D) were conducted. Following exposure to initial heat shock, Jurkat cells treated with 50 and 100µM resveratrol showed a statistically significant reduction ( $p<0.05$ ) in caspase-3 activity (B), and U937 cells treated with 25µM ( $p<0.05$ ) and 100µM ( $p<0.01$ ) resveratrol a statistically significant increase in cell viability (C) compared with control cells. No statistically significant difference in cell viability in Jurkat cells (A) or caspase-3 activity in U937 cells (D) was seen. Values are shown as mean  $\pm$  SEM of samples performed in triplicate or quadruplicate. Statistical analysis was conducted using two-tailed unpaired Student's  $t$ -tests. Heat shock treated cells versus control cells: \*  $p<0.05$ , \*\*  $p<0.01$ .

Jurkat and U937 cells were pre-treated with heat shock for 1 hr at 42°C followed by a 3 hr recovery before incubation with various concentrations of resveratrol for 20 hrs. Pre-treatment of Jurkat cells to heat shock caused a small but significant reduction in sensitivity to resveratrol-induced cell death ( $p<0.05$  at 50 and 100µM resveratrol; figure 3.12A). A small but significant reduction in sensitivity to resveratrol-induced apoptosis was detected in Jurkat cells with the caspase-3 assay ( $p<0.05$ ; figure 3.12B), and to resveratrol-induced cell death in U937 as measured with the PI assay ( $p<0.05$ ; figure 3.12C). This effect was not statistically significant for the PI assay in Jurkat cells or for the caspase-3 assay in U937 cells (figure 3.12A and D).

### **3.5.2. Suppressing Hsp72 expression by pre-treating cells to ethanol prior to resveratrol treatment is insufficient to sensitise cells to resveratrol-induced cell death.**

A time course of treatment of Jurkat and U937 cells with 1.25% ethanol was conducted in order to determine parameters to inhibit HSP expression without causing cell death. A concentration of 1.25% ethanol was used based on previously successful results with this concentration (communication with Nina Dempsey) and it was previously ascertained that a 24 hr treatment of cells with this concentration did not cause significant cell death (figure 3.7). A 2 hr treatment of Jurkat cells with 1.25% ethanol was found to cause the largest decrease in intracellular Hsp72 ( $p < 0.05$ ; figure 3.13). This data was obtained in collaboration with Nina Dempsey.

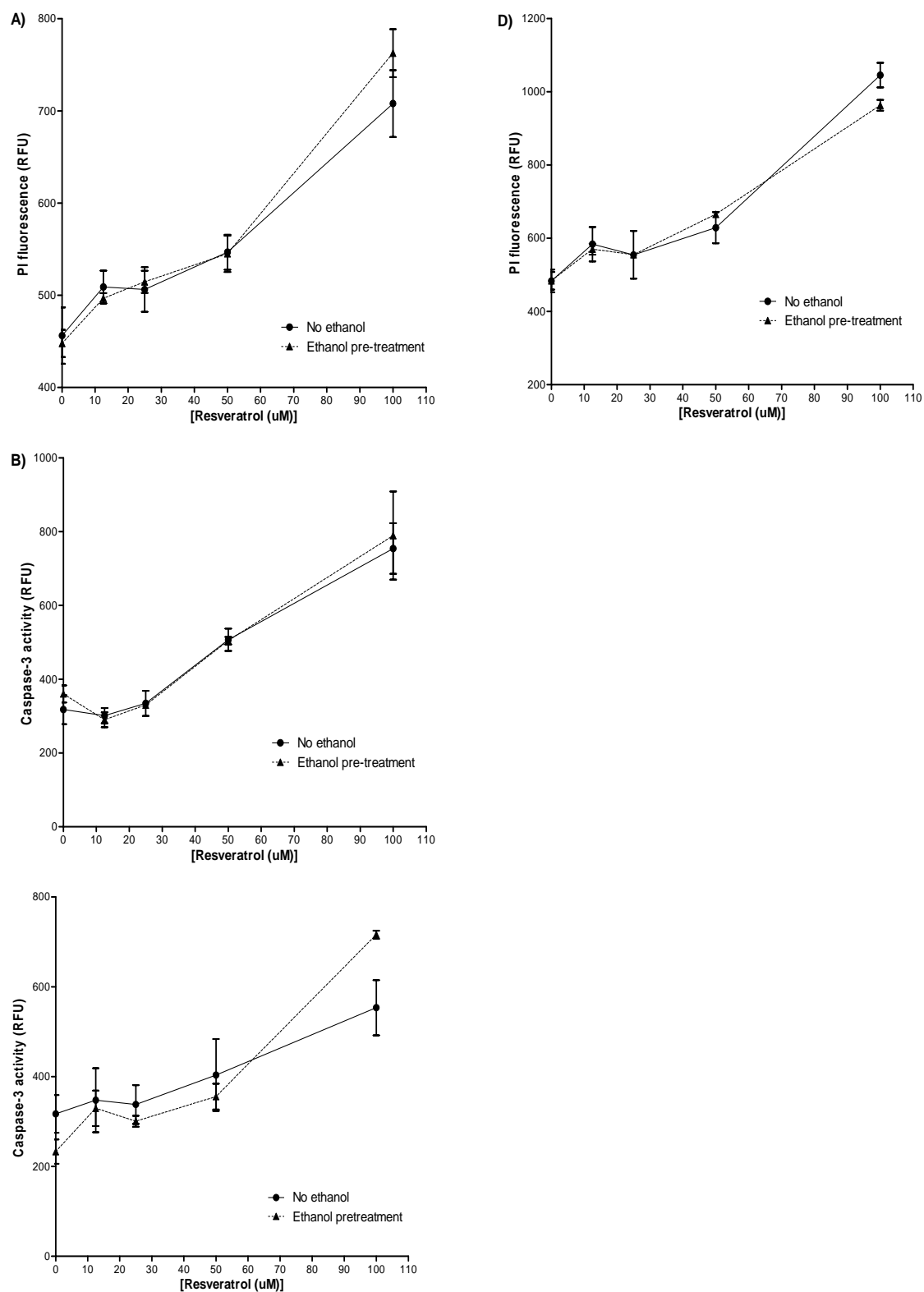




**Figure 3. 13.** *The effect of ethanol on levels of intracellular Hsp72 (iHsp72) in Jurkat cells.*

Jurkat cells were treated with 1.25% ethanol for various durations and levels of iHsp72 analysed by anti-Hsp72 binding with flow cytometry. A significant decrease in iHsp72 levels from untreated cells was seen after 2 and 4 hours incubation with ethanol ( $p < 0.05$ ), the greatest reduction being after 2 hours. After 4 hours incubation, the levels of iHsp72 increased to similar levels as untreated cells at 5 hours incubation. Values are shown as mean  $\pm$  SEM of samples performed in triplicate. Statistical significance was analysed using one-way ANOVA and post-hoc Dunnett test. Treated cells versus untreated cells: \*  $p < 0.05$ . These data were collected in collaboration with Nina Dempsey.

Jurkat and U937 cells were pre-treated with 1.25% ethanol for 2 hrs before incubation with various concentrations of resveratrol for 20 hrs. Pre-treatment with ethanol had no effect on the sensitivity of cells to resveratrol-induced cell death in either cell line (figure 3.14A-D).



**Figure 3.14.** *Pre-treatment of cells with ethanol had no effect on sensitivity of cells to resveratrol-induced cell death.*

Jurkat (A, B) and U937 (C, D) cells were incubated with various concentrations of resveratrol for 20 hours following an initial expose to ethanol (2 hour incubation with 1.25% ethanol) or no exposure to ethanol. After treatment, a PI assay (A, D) and caspase-3 assay (B, C) were conducted. No significant differences in cell viability or caspase-3 activity were seen in Jurkat or U937 cells between cells exposed to ethanol and not (A-D). Values are shown as mean  $\pm$  SEM of samples done in triplicate or quadruplicate. Statistical analysis was conducted using two-tailed unpaired Student's *t*-tests.

## 4. Discussion

Resistance of cancer cells to apoptosis remains a major obstacle to the efficacy of anticancer treatments, and the identification of methods to decrease or counteract this resistance is paramount to the progression of cancer management.

Resveratrol has gained much attention for its anti-cancer properties. Research has demonstrated its chemopreventive activity and investigating its potential as a chemotherapeutic agent (Signorelli and Ghidoni, 2005). Resveratrol has been found to cause cell cycle arrest and induce apoptosis of a variety of different cancer cell lines *in vitro* and reduce tumour mass in animal models *in vivo* (Jang et al., 1997; Garvin et al., 2006; Kalra et al., 2008; Clément et al., 1998; Bernhard et al., 2000; Dörrie et al., 2001; Delmas et al., 2003). However, the true clinical potential of resveratrol is unknown. It will depend on the concentration needed for therapeutic efficacy, its bioavailability, and its level of toxicity. Relatively high concentrations of resveratrol may be needed for effective apoptosis of certain cancer cell lines (Wang et al., 2005), which may increase the risk of toxicity and be difficult to achieve *in situ*. Thus, it is useful to consider the use of resveratrol in combination with other anticancer agents/therapies that allow lower concentrations to be needed for efficacy.

TRAIL is a promising anti-cancer agent that induces apoptosis specifically in cancer cells, although resistance is also common (Clemons and Anderson, 2006). Ethanol has also been shown to induce apoptosis and sensitise cancer cells to TRAIL-induced

apoptosis (Vacluova et al., 2004). Both TRAIL and ethanol are relevant for use in combination therapy with resveratrol. Resveratrol has been observed to sensitise cells to TRAIL-induced apoptosis previously, and ethanol is a constituent of red wine and often used as a vector or solvent for other drugs. Both ethanol and resveratrol have been shown to cause lipid raft aggregation, but may differ in their effect on downstream signalling events (Delmas et al., 2004; Vaculová et al., 2004; Fulda and Debatin, 2005). No previous studies have investigated the effect of alcohol and resveratrol in combination with the aim to induce apoptosis, or the combination effect on TRAIL-induced apoptosis despite that the major source of resveratrol in the Western diet is from alcoholic red wine (Baur and Sinclair, 2006).

Aggregation of lipid rafts is closely linked with the heat shock response, as changes in the lipid domain may signal a stress response and induce the expression of Hsps. Hsps are often elevated in cancer cells, including leukaemia cells (Schmitt et al., 2007; Lanneau et al., 2008). Intracellular Hsp72 has been shown to be a strongly anti-apoptotic and confer cancer cells with resistance to apoptosis (Clemons and Anderson, 2006). On the other hand, surface Hsp70 displays immunological properties and may induce elimination of cancer cells. Thus, therapies to reduce intracellular Hsp70 levels and increase surface/extracellular Hsp70 have shown promise in sensitising cancer cells to apoptosis (Lanneau et al., 2008; Rashmi et al., 2004).

Bearing the above in mind, studies were designed to: 1) investigate the effect of resveratrol on two types of leukaemia cells *in vitro* alone or in combination with

ethanol, TRAIL or both, and 2) investigate the effect of resveratrol on both intracellular and surface Hsp72 expression and whether up- or downregulating Hsp72 expression can alter the cytotoxic effect of resveratrol.

#### ***4.1. Induction of cell death by resveratrol occurs by apoptosis or necrosis depending on concentration***

In this study, a 24 hour incubation with resveratrol caused dose-dependent cell death in leukaemia cell lines Jurkat and U937 in the concentration range of 50 $\mu$ M and above. Cell death was indicated as late apoptosis or necrosis by disruption of membrane integrity measured with the PI assay and reduction in mitochondrial activity measure by the MTS assay. Apoptosis was indicated by a rise in caspase-3 activity and was significantly induced at 100-200 $\mu$ M resveratrol, but sharply declined above 200 $\mu$ M, suggesting a transition from apoptosis to necrosis occurred at the higher concentrations. The results were confirmed by FACS analysis using annexin-V presentation and PI staining.

These and similar assays have been used in previous studies investigating the effect of resveratrol on cell viability and apoptosis of various cell types. Limitations of the assays include the ability of the PI and MTS assays only to detect features of cell death and caspase-3 assay only to detect levels of a specific caspase generally involved in apoptosis. However, they are considered to be valid and reliable assays. The results gain validity by confirmation with FACS analysis which is able to better characterise

the cell population by measuring parameters involved in early and late apoptosis and necrosis. Resveratrol has been shown to induce apoptosis via caspase-3 activation in U937 cells previously (Park et al., 2001). However, research by Bernhard et al. (2000) suggested that caspase-6 was the essential caspase while caspase-3 was activated to a much lesser extent in CEM-C7H2 and Jurkat cells.

It is worth noting that the lowest concentration (50 $\mu$ M) of resveratrol that induced significant cell death as detected in the PI and MTS assays did not correlate with an increase in caspase-3 activity. Thus, what form of cell death was induced by 50 $\mu$ M resveratrol? It was assumed to be apoptosis due to existing literature indicating that apoptosis is likely to occur at the concentration, and because apoptosis was shown to occur at higher concentrations followed by a shift to necrosis only at the highest concentrations of resveratrol used. The lack of significant caspase-3 activity at 50 $\mu$ M resveratrol could be due to increased caspase-3 activity not remaining elevated for detection after 24 hours treatment, or other flaws with the experiment. It is also possible that apoptosis was independent of caspase-3 at the lower concentrations of resveratrol. It is certain that the results would have benefitted from an increased number of repeats and review of the methodology to validate the result.

Efficacious concentrations of resveratrol to induce apoptosis have previously been found to be ~20–100 $\mu$ M after 8 – 72 hours incubation (Surh et al., 1999; Clément et al., 1998; Gescher & Steward, 2003; Dörrie et al., 2001; Ferry-Dumanez et al., 2002; Cecchinato et al., 2007; Bernhard et al., 2000; Park et al., 2001). It is difficult to

determine the lowest effective dose due to differing or lacking statistical methods used to determine significance.

In contrast to apoptosis induction, Ferry-Dumanez et al. (2002) reported that 50% growth inhibition during 3 days incubation was achieved with 13.42 $\mu$ M and 16.09 $\mu$ M resveratrol in Jurkat and U937 cells, respectively. Hence, the cell lines were shown to be similar in sensitivity to resveratrol and display greater sensitivity than several other leukaemia cell lines (Ferry-Dumanez et al., 2002; Dörrie et al., 2001).

Thus, the findings of this study generally comply with the results of previous studies in demonstrating that Jurkat and U937 cells are of similar sensitivity to resveratrol-induced apoptosis and that this occurs at 50-200 $\mu$ M resveratrol after 24 hours incubation, notably a slightly higher concentration range than previously reported, and other studies have noted necrosis as the main form of cell death at 200 $\mu$ M (Cardile et al., 2003). The FACS analysis in this study only included up to 100 $\mu$ M resveratrol; it would have been interesting to use higher concentrations to further investigate the transition from apoptosis to necrosis.

It is worth noting that research exists that indicates that Jurkat cells are relatively resistant to resveratrol-induced apoptosis (Wang et al., 2005), contrary to the presented results. It is difficult to explain this discrepancy since the presented study and others previously have demonstrated the efficacy of resveratrol-induced cell death in Jurkat



cells. It is proposed that a different type of Jurakt cells may have been used that lacked a vital mechanism for resveratrol-induced cell death.

Whereas only dose-dependent activity was demonstrated here, other studies have demonstrated time-dependent activity also, showing an increase in resveratrol-induced growth arrest and apoptosis after 24, 48, 72 and 96 hours incubation (Clément et al., 1998; Dörrie et al., 2001; Fulda and Debatin, 2004). Other time points than 24 hours were not included in this study since significant apoptosis was shown to occur, shorter incubation times proved less efficacious (data not shown), and longer incubation times were not considered beneficial to the findings.

#### ***4.2. Resveratrol sensitizes leukaemia cells to TRAIL-induced apoptosis and displays a synergistic effect with ethanol on cell death and TRAIL-induced apoptosis***

Combination treatments with resveratrol and sub-lethal concentrations of ethanol or TRAIL showed synergistic effects on dose-dependent cell death. The inclusion of the highest non-lethal concentration of ethanol (1.25%) abolished any additional apoptotic effect of TRAIL, suggesting that the concentration was relatively toxic. A lower, less toxic concentration of ethanol was therefore selected for further investigations (0.5%) and unmasked an additive effect on cell death with each of the three compounds included in the treatment. Thus, treatment with all compounds together (resveratrol,

ethanol and TRAIL) showed the greatest efficacy with significantly greater apoptotic cell death occurring at 12.5 and 25 $\mu$ M resveratrol than the other treatments. However, this triple treatment also showed the greatest toxicity with a progression from apoptosis to necrosis occurring at a lower concentration of resveratrol (25-50 $\mu$ M) than the other combination treatments (100-200 $\mu$ M). This toxicity may be cause for concern. However, it is promising that despite displaying much lower toxicity the double combination treatments with ethanol or TRAIL caused significantly higher apoptosis than resveratrol alone at low concentrations (25–100 $\mu$ M) resveratrol. This shows promise for the use of resveratrol as a sensitising agent for TRAIL-induced apoptosis and highlights that apoptosis may be enhanced by resveratrol and ethanol in combination. It may be feasible to overcome some toxicity of the triple treatment by using pre-treatments of some of the compounds in stead of combination treatments, e.g. Delmas et al. (2004) and Fulda and Debatin (2004) used 24 hour pre-treatments with resveratrol in colon cancer cells or Jurkat cells before adding a further anticancer agent (TRAIL and AraC, respectively) and showed that resveratrol significantly sensitised the cells to apoptosis.

In summary, the results presented in this section confirm previous reports that resveratrol sensitises Jurkat cells to TRAIL-induced apoptosis (Fulda and Debatin, 2005), and reports for the first time that this is also the case in U937 cells. Whilst Fulda and Debatin (2005) required 10-30ng/mL TRAIL and 100 $\mu$ M resveratrol to see a significant increase in apoptosis, this study shows that with 25ng/mL TRAIL only 25 $\mu$ M resveratrol was required for a significantly raised apoptotic effect. Delmas et al.

(2004) observed a sensitising effect with 10-30 $\mu$ M resveratrol, but this was after a 48 hr incubation and with 100ng/mL TRAIL which was believed to be cytotoxic on its own in the cells used. Thus, the results presented appear of equivalent or better efficacy than previously reported.

This study highlights for the first time that the combined use of resveratrol and ethanol displays an enhanced apoptotic effect without excessive toxicity compared with TRAIL combination therapy. Significantly enhanced apoptosis was achieved with 0.5% ethanol at as low as 25 $\mu$ M resveratrol compared with resveratrol alone. These concentrations are significantly lower than those used by Vaculová et al. (2004) who used 4% ethanol and 100ng/ml TRAIL to show a significant sensitising effect of ethanol on TRAIL-induced apoptosis in colon cancer cells. However, colon cancer cells are known to be relatively resistant to ethanol and leukaemia cells based on the data presented appear to be more sensitive. Nourissat et al. (2008) observed lipid raft clustering with 50mM (~0.29%) ethanol. Thus, it is reasonable to assume that lipid raft clustering may occur at 0.5% ethanol unless there are vast differences in the effects produced in the different cell types.

The mechanism behind the synergistic effect of ethanol and resveratrol was not investigated. However, ethanol was shown in later results to depress Hsp72 levels which may in part have enhanced the apoptotic effect of resveratrol. However, there are likely to be other mechanisms involved considering that ethanol and resveratrol both modify lipid raft aggregation, but display differing effects on downstream

signalling (i.e. resveratrol may depend on mitochondria for apoptosis induction and ethanol not), and are both able to sensitise cells to TRAIL-induced apoptosis. Thus, the mechanisms behind the synergy warrant further investigation.

It is important to note that resveratrol has previously been investigated as a compound with promise to reduce ethanol toxicity, the opposite aim of the current study. Chan and Chang (2006) presented such a study using K562 leukaemia cells. Using incubations of 200-300mM (11.5-17.25%) ethanol they observed that low concentrations of resveratrol (10-25 $\mu$ M) reduced apoptosis and may be protective against ethanol-induced toxicity, but higher (100-200 $\mu$ M) concentrations of resveratrol enhanced apoptosis and produced a combined increased cytotoxic effect. This study highlights differing effects occurring from different concentrations used. Chan and Chang (2006) used 20-35-fold higher concentrations of ethanol than the current study but required 100 $\mu$ M resveratrol to induce augmented apoptosis and therefore does not agree with the presented findings in which enhanced apoptosis was achieved with 0.5% ethanol and 25 $\mu$ M resveratrol. This may be due to the vastly different concentrations of ethanol and the different cell line used, as K562 cells have been shown to be more resistant to resveratrol than Jurkat and U937 cells (Ferry-Dumazet et al., 2002).

It would have been interesting to have investigated the level of apoptosis induced by ethanol and TRAIL alone to compare the sensitizing effects of resveratrol and ethanol on TRAIL-induced apoptosis. Resveratrol is considered to be less toxic than ethanol, although efficacious concentrations may be harder to achieve *in vitro*.

It is important to note that although 0.5% ethanol (equivalent to 0.4g/dl) is not toxic to leukaemia cells *in vitro*, equivalent concentrations in human blood have been linked with acute alcohol poisoning (Jones and Holmgren, 2003). Vonghia et al. (2008) report that blood alcohol concentrations as low as 0.05g/dl can cause change in behaviour. This highlights the unsuitability of the concentrations of ethanol used here to *in vivo* or clinical use, but is valuable by showing the potential of ethanol or a similar-acting compound and shows efficacy with lower concentrations than that used in studies previously.

#### ***4.3. Effect of resveratrol on Hsp72 expression***

In this study, 12.5-50 $\mu$ M resveratrol caused raised levels of intracellular Hsp72 compared with untreated control. The elevation peaked at 25 $\mu$ M and declined at 50 $\mu$ M. In previous results, these low concentrations failed to demonstrate caspase-3 dependent apoptosis although 50 $\mu$ M caused significant cell death as indicated by the PI and MTS assays. Elevation of intracellular Hsp72 may have protected the cells from apoptosis at low concentrations of resveratrol, whereas higher concentrations caused no change to or decreased Hsp72 levels at the top concentration of 200 $\mu$ M and thus allowed apoptosis to occur. Above 200 $\mu$ M resveratrol, a shift from apoptosis to necrosis would cause a loss of intracellular Hsp72 such that resveratrol may not cause depletion of intracellular levels of Hsp72 in intact cells.

These findings are the first of their kind in leukaemia cell lines and the intracellular Hsp72 findings agree with related results presented by Cardile et al. (2003) and Putics et al. (2008). Cardile et al. (2003) reported that 50-100 $\mu$ M resveratrol induced Hsp70 expression but 200 $\mu$ M caused no change in levels compared with control in human DU-145 prostate cancer cells, showing a similar pattern to the result presented here although with a higher range of resveratrol concentrations. Putics et al. (2008) reported that resveratrol activated HSF-1 and expression of Hsp70 in peripheral lymphocytes, monkey fibroblasts and human cervical cancer HeLa cells, but did not show that Hsp70 levels were significantly reduced at higher concentrations of resveratrol although activation was seen to level off at 100-200 $\mu$ M resveratrol.

The presented results are in contrast to findings by Chakraborty et al. (2008) who observed that 40 $\mu$ M resveratrol reduced Hsp70 levels over 24-72 hours in K562 leukaemia cells with corresponding increases in apoptosis. This might imply that K562 cells could be more sensitive to resveratrol. However, Ferry-Dumazet et al. (2002) observed that K562 cells were more resistant to the anti-proliferative effect of resveratrol than Jurkat and U937 cells, but would also undergo apoptosis. This research group also showed that resveratrol has different effects on cell cycle changes and cyclins in different leukaemia cell lines highlighting the complex cell dependent alterations induced by resveratrol.

Studies investigating the effect of resveratrol on surface expression of Hsp72 were not found in literature searches accompanying this study, and thus the results presented are

believed to be so for the first time. Resveratrol caused a dose-dependent increase in surface levels of Hsp72. The finding that resveratrol increased Hsp72 at all concentrations used could indicate an induction of Hsp72 at lower, non-lethal concentrations (as observed by Putics et al., 2008 in other cell lines) and a movement of newly made proteins to the cell membrane, possibly to act as danger signals to the extracellular environment whilst elevated intracellular levels protect the cell. The level of surface Hsp72 levelled off at 100 $\mu$ M resveratrol when relative risk of apoptosis was high and the further increase at 200 $\mu$ M may reflect the shift to necrosis that is likely to occur at this concentration and thus, leakage of intracellular proteins. A shift from intracellular to extracellular Hsp72 at intermediate resveratrol concentrations may indicate a shift in danger management from cytoprotection to extracellular signalling.

It is interesting that the induced expression of Hsp72 is not contained to intracellular protection purposes but is transported to the cell surface where it may play a role in stimulating the innate immune system. Furthermore, the findings indicating that intracellular Hsp72 levels may be protective of apoptosis suggest that resveratrol-induced apoptosis may be more effective if Hsp72 levels were depressed. Hence, Hsp treatment is likely to be relevant to resveratrol efficacy as an anticancer agent.

#### ***4.4. Manipulation of intracellular levels of Hsp72 may affect sensitivity of leukaemia cells to resveratrol-induced cell death***

In this study, increasing intracellular Hsp72 expression with a mild heat shock prior to resveratrol treatment caused a slight but significant increase in resistance of Jurkat and U937 cells to resveratrol-induced cell death. Significant results were seen at 50-100 $\mu$ M resveratrol with the caspase-3 assay only in Jurkat cells, and at 25 and 100 $\mu$ M with the PI assay only in U937 cells. Although a similar trend was seen in the PI assay for Jurkat cells and caspase-3 assay in U937 cells, the results were not significant, indicating that any changes were relatively small and thus difficult to obtain statistical significance for. It is possible that the change in sensitivity may have been more apparent at an earlier time point than 24 hours. However, statistical significance was achieved providing a novel finding that indicates that elevating Hsp72 levels does increase the resistance of Jurkat and U937 cells to resveratrol-induced cell death. Whilst Putics et al. (2008) showed that resveratrol synergized with mild-moderate heat shock, conferring enhanced cytoprotection against severe heat stress it is shown here that this effect extends to cytoprotection against resveratrol itself.

Lowering intracellular Hsp72 expression by treatment with ethanol prior to resveratrol treatment was insufficient to increase sensitivity to resveratrol-induced cell death. However, there are several limitations to the experiment that may discredit the validity of this negative result, and the possibility that a true positive effect exists should not be dismissed. The limitations include that ethanol treatment is not a validated method to



depress Hsp72, the experimental procedure included steps that may have induced Hsp72 expression after ethanol treatment, and the experiments was only conducted once due to time restrictions and repeating the experiment to generate sufficient *n* numbers and improve statistical power would improve validity. Whilst results are presented demonstrating that 1.25% ethanol caused a significant reduction of intracellular Hsp72 after 2 hours incubation, the many steps in the experimental procedure after this initial incubation until resveratrol treatment was commenced (e.g. centrifugation, washing and re-suspending of cells causing possible mechanical stress and changes in temperature, although conducted with as much care and speed as possible) could have affected Hsp72 induction and uncoupled the effect of alcohol treatment. In addition, cells were incubated with resveratrol for 24 hours whereas the effect of depressed Hsp72 levels may only have lasted a shorter time period up until resveratrol was able to induce HSF-1 activity. Addressing the limitations to the experimental procedure to produce results with greater validity would be of great interest to elucidate whether Hsp72 reduction could increase the efficacy of resveratrol-induced apoptosis. It would be especially interesting if low concentrations of resveratrol could be found to be effective as these may be the more relevant for *in vivo*/clinical work.

#### ***4.5. Further research and clinical relevance***

The presented findings confirm that resveratrol induces apoptosis and sensitises cells to TRAIL-induced apoptosis, and further reports that resveratrol acts synergistically with ethanol to enhance apoptosis in leukaemia cells *in vitro*. The mechanisms of these effects have yet to be fully elucidated, and warrant further investigation. Crucially, further research is required to assess whether these findings have clinical relevance. It is thus necessary to investigate what concentrations of resveratrol can be achieved *in vivo* and what concentrations are needed for therapeutic efficacy. Included in this, the toxicity profile and bioavailability need to be elucidated.

Studies investigating the bioavailability of resveratrol have so far suggested that it is rapidly metabolised into its conjugates in the human body, and that these should therefore be considered in future studies (Wenzel and Somoza, 2005). It is thus necessary to characterize the therapeutic potential and efficacy of the conjugates and whether they have potential in anticancer therapy and if not, if it is possible to achieve suitable concentrations of unmodified resveratrol for clinical efficacy. Quercetin, a polyphenol found in many similar dietary sources as resveratrol, has been shown to increase the bioavailability of resveratrol by inhibiting relevant enzymes and thus is worthwhile further investigation (De Santi et al., 2000).

Due to the predicted difficulty of achieving efficacious concentrations of resveratrol *in vivo*, research investigating further methods to enhance its efficacy is of interest. For

example, other dietary polyphenols may synergize with resveratrol and be potentially less toxic than ethanol. Quercetin has been shown to cause lipid raft aggregation and sensitise cells to TRAIL-induced apoptosis as well as increasing the bioavailability of resveratrol (Psahoulia et al., 2007). Curcumin is another polyphenol with promising anticancer and anti-inflammatory activity that may be worth testing in combination with resveratrol. Interestingly, Hsp70 modulation has recently been shown to change the sensitivity of colon cancer cells to curcumin-induced apoptosis (Rashmi et al., 2004). Crucially, the potential of Hsp72 manipulation to modulate efficacy of resveratrol was highlighted in this study.

The findings present several questions regarding the involvement of Hsp70 in the mechanisms that resveratrol exhibits. Low concentrations of resveratrol that induce Hsp72 expression have been shown to be cytoprotective (Chan and Chang, 2006) whilst higher concentrations that fail to stimulate Hsp72 expression are cytotoxic, suggesting that Hsp induction could be the link between the cytoprotective and cytotoxic effects of resveratrol. Further investigation into this area may develop our understanding of resveratrol and Hsps and how they may be used for clinical benefit. For example, by modulating Hsp72 would it be possible to augment and control the cyto-protective/toxic effect of resveratrol? It would also be interesting to investigate whether the enhanced surface level expression of Hsp72 induced by resveratrol could activate the innate immune system *in situ* resulting in enhanced elimination of cancer cells (and override the anti-apoptotic effect conferred by increased intracellular Hsp72). If so, resveratrol may prove more efficacious *in situ* than *in vitro* and Hsp

therapy to reduce intracellular Hsp72 and increase surface Hsp72 levels would appear promising in combination anticancer therapy.

Although pharmacological levels of the compounds have been discussed in this study, it would be of further interest to investigate whether oral consumption of dietary sources containing synergistic compounds (e.g. resveratrol, quercetin and ethanol in red wine) could be used to sensitise cancer cells *in situ* to anticancer therapy. I.e. could red wine or grape juice consumption render traditional or novel anticancer therapies more efficacious?

## 5. Conclusions

- Resveratrol induces cell death in the lymphoid and myeloid leukaemia cell lines Jurkat and U937.
- Induced cell death is in the form of apoptosis at lower concentrations, and necrosis at higher concentrations, of resveratrol.
- Resveratrol sensitises the leukaemia cell lines to TRAIL-induced apoptosis and displays a synergistic effect with ethanol on apoptosis induction.
- Resveratrol, ethanol and TRAIL act synergistically in induction of apoptosis and cell death. Combination treatment with all three compounds induces a highly efficacious apoptosis at a lower concentration of resveratrol than achieved with single or double treatments, but also shows a greater potential toxicity by transition from apoptosis to necrosis occurring at lower concentrations of resveratrol.
- Hsp72 expression is induced by low concentrations of resveratrol that are not effective at inducing apoptosis, revealed by a rise in intracellular and extracellular Hsp72 levels. Intracellular Hsp72 levels remain unchanged or reduced by higher concentrations of resveratrol that are able to induce significant apoptosis, whilst surface Hsp72 is further increased at these concentrations.
- Increasing intracellular Hsp72 levels with a mild heat shock confers cells with increased resistance to resveratrol-induced apoptosis/cell death.
- It was not possible to show that decreasing intracellular Hsp72 levels with ethanol sensitise cell to resveratrol-induced apoptosis. However, since the experimental

procedure was flawed, the validity of the results is questioned and further investigation believed worthwhile.

- Further research should consider how resveratrol treatment may gain clinical relevance by investigating how therapeutic concentrations may be achieved *in vivo*, whether other compounds with synergizing effects may benefit therapy, consider the impact of Hsp72 modulation, and whether pharmacological levels are required or whether oral consumption of dietary sources could enhance anticancer therapy.

## 6. References

Athar, M., Back, J. H., Tang, X., Kim, K. H., Kopelovich, L., Bickers, D. R., & Kim, A. L. (2007). Resveratrol: A review of preclinical studies for human cancer prevention. *Toxicology & Applied Pharmacology*, 224(3), 274-283.

Aziz, M. H., Nihal, M., Fu, V. X., Jarrad, D. F., & Ahmad, N. (2006). Resveratrol-caused apoptosis of human prostate carcinoma LNCaP cells is mediated via modulation of phosphatidylinositol 3'-kinase / Akt pathway and Bcl-2 family proteins. *Molecular Cancer Therapeutics*, 5(5), 1335-1341.

Balogh, G., Horváth, I., Nagy, E., Hoyk, Z., Benkő, S., Bensaude, O., & Vigh, L. (2005). The hyperfluidization of mammalian cell membranes acts as a signal to initiate the heat shock protein response. *FEBS Journal*, 272(23), 6077-6086.

Baur, J. A., & Sinclair D. A. (2006). Therapeutic potential of resveratrol: the in vivo evidence. *Nature Reviews. Drug Discovery*, 5(6), 493-506.

Bernhard, D., Tinhofer, I., Tonko, M., Hübl, H., Ausserlechner, M. J., Greil, R., Kofler, R., & Csordas, A. (2000). Resveratrol causes arrest in the S-phase prior to Fas-independent apoptosis in CEM-C7H2 acute leukaemia cells. *Cell Death and Differentiation*, 7(9), 834-842.

Cardile, V., Scifo, C., Russo, A., Falsaperla, M., Morgia, G., Motta, M., Renis, M., Imbriani, E., & Silvestre, G. (2003). Involvement of HSP70 in resveratrol-induced apoptosis of human prostate cancer. *Anticancer Research*, 23(6C), 4921-4926.

Carratù, L., Franceschelli, S., Pardini, C. L., Kobayashi, G. S., Horváth, I., Vigh, L., & Maresca, B. (1996). Membrane lipid perturbation modifies the set point of the temperature of heat shock response in yeast. *Proceedings of the National Academy of Sciences U S A*, 93(9), 3870-3875.

Cecchinato, V., Chiaramonte, R., Nizzardo, M., Cristofaro, B., Basile, A., Sherbet, G. V., & Comi, P. (2007). Resveratrol-induced apoptosis in human T-cell acute lymphoblastic leukaemia MOLT-4 cells. *Biochemical Pharmacology*, 74(11), 1568-1574.

Chakraborty, P. K., Mustafi, S. B., Ganguly, S., Chatterjee, M., & Raha, S. (2008). Resveratrol induces apoptosis in K562 (chronic myelogenous leukemia) cells by targeting a key survival protein, heat shock protein 70. *Cancer Science*, 99(6), 1109-1116.

Chan W. H., & Chang Y. J. (2006). Dosage effects of resveratrol on ethanol-induced cell death in the human K562 cell line. *Toxicology Letters*, 161(1), 1-9.



Clemens, N. J., & Anderson, R. L. (2006). TRAIL-induced apoptosis is enhanced by heat shock protein 70 expression. *Cell Stress & Chaperones*, 11(4), 343-355.

Clément, M. V., Hirpara, J. L., Chawdhury, S. H., & Pervaiz, S. (1998). Chemopreventive agent resveratrol, a natural product derived from grapes, triggers CD95 signaling-dependent apoptosis in human tumor cells. *Blood*, 92(3), 996-1002.

Delmas, D., Rébé, C., Lacour, S., Filomenko, R., Athias, A., Gambert, P., Cherkaoui-Malki, M., Jannin, B., Dubrez-Daloz, L., Latruffe, N., & Solary, E. (2003). Resveratrol-induced apoptosis is associated with Fas redistribution in the rafts and the formation of a death-inducing signaling complex in colon cancer cells. *Journal of Biological Chemistry*, 278(42), 41482-41490.

Delmas D, Rébé C, Micheau O, Athias A, Gambert P, Grazide S, Laurent G, Latruffe N, Solary E. (2004). Redistribution of CD95, DR4 and DR5 in rafts accounts for the synergistic toxicity of resveratrol and death receptor ligands in colon carcinoma cells. *Oncogene*, 23(55), 8979-8986.

Delmas, D., Lançon, A., Colin, D., Jannin, B., & Latruffe, N. (2006). Resveratrol as a chemopreventive agent: a promising molecule for fighting cancer. *Current Drug Targets*, 7(4), 423-442.

De Santi, C., Pietrabissa, A., Spisni, R., Mosca, F., & Pacifici, G. M. (2000). Sulphation of resveratrol, a natural product present in grapes and wine, in the human liver and duodenum. *Xenobiotica*, 30(6), 609-617.

Dörrie, J., Gerauer, H., Wachter, Y., & Zunino, S. J. (2001). Resveratrol induces extensive apoptosis by depolarizing mitochondrial membranes and activating caspase-9 in acute lymphoblastic leukemia cells. *Cancer Research*, 61(12), 4731-4739.

Ferry-Dumazet, H., Garnier, O., Mamani-Matsuda, M., Vercauteren, J., Belloc, F., Billiard, C., Dupouy, M., Thiolat, D., Kolb, J. P., Marit, G., Reiffers, J., & Mossalayi, M. D. (2002). Resveratrol inhibits the growth and induces the apoptosis of both normal and leukemic hematopoietic cells. *Carcinogenesis*, 23(8), 1327-1333.

Fulda, S., & Debatin, K. M. (2004). Sensitization for anticancer drug-induced apoptosis by the chemopreventive agent resveratrol. *Oncogene*, 23, 6702–6711.

Fulda, S., & Debatin, K. M. (2005). Resveratrol-mediated sensitisation to TRAIL-induced apoptosis depends on death receptor and mitochondrial signalling. *European Journal of Cancer*, 41(5), 786-798.

Fulda, S., & Debatin, K. M. (2006). Resveratrol modulation of signal transduction in apoptosis and cell survival: A mini-review. *Cancer Detection and Prevention*, 30(3), 217-223.

Garrido, C., Gurbuxani, S., Ravagnan, L., & Kroemer, G. (2001). Heat shock proteins: endogenous modulators of apoptotic cell death. *Biochemical & Biophysical Research Communications*, 286(3), 433-442.

Garvin, S., Ollinger, K., & Dabrosin, C. (2006). Resveratrol induces apoptosis and inhibits angiogenesis in human breast cancer xenografts in vivo. *Cancer Letters*, 231, 113–122.

Gastpar, R., Gehrmann, M., Bausero, M. A., Asea, A., Gross, C., Schroeder, J. A., & Multhoff, G. (2005). Heat shock protein 70 surface-positive tumor exosomes stimulate migratory and cytolytic activity of natural killer cells. *Cancer Research*, 65(12), 5238-5247.

Gautam, S. C., Xu, Y.X., Dumaguin, M., Janakiraman, N., & Chapman, R. A. (2000). Resveratrol selectively inhibits leukemia cells: a prospective agent for ex vivo bone marrow purging. *Bone Marrow Transplant*, 25(6), 639-645.

Gehrmann, M., Pfister, K., Hutzler, P., Gastpar, R., Margulis, B., Multhoff, G. (2002). Effects of antineoplastic agents on cytoplasmic and membrane-bound heat shock protein 70 (Hsp70) levels. *Journal of Biological Chemistry*, 383(11), 1715-1725.

Gehrmann, M., Brunner, M., Pfister, K., Reichle, A., Kremmer, E., & Multhoff G. (2004). Differential up-regulation of cytosolic and membrane-bound heat shock protein 70 in tumor cells by anti-inflammatory drugs. *Clinical Cancer Research*, 10(10), 3354-3364.

Gescher, A. J., & Steward, W. P. (2003). Relationship between Mechanisms, Bioavailability, and Preclinical Chemopreventive Efficacy of Resveratrol: A Conundrum. *Cancer Epidemiology Biomarkers & Prevention*, 12, 953-957.

Goldberg, D. M, Yan, J., Soleas, G. J. (2003). Absorption of three wine-related polyphenols in three different matrices by healthy subjects. *Clinical Biochemistry*, 36(1), 79-87.

Han, Y. S., Zheng, W. H., Bastianetto, S., Chabot, J. G., & Quirion, R. (2004). Neuroprotective effects of resveratrol against beta-amyloid-induced neurotoxicity in rat hippocampal neurons: involvement of protein kinase C. *British Journal of Pharmacology*, 141(6), 997-1005.

Han, Y. S., Bastianetto, S., Dumont, Y., & Quirion, R. (2006). Specific plasma membrane binding sites for polyphenols, including resveratrol, in the rat brain. *Journal of Pharmacological & Experimental Therapeutics*, 318(1), 238-245.

Hayashibara, T., Yamada, Y., Nakayama, S., Harasawa, H., Tsuruda, K., Sugahara, K., Miyanishi, T., Kamihira, S., Tomonaga, M., & Maita, T. (2002). Resveratrol induces downregulation in survivin expression and apoptosis in HTLV-1-infected cell lines: a prospective agent for adult T cell leukemia chemotherapy. *Nutrition and Cancer*, 44(2), 192-201.

Horváth, I., Glatz, A., Varvasovszki, V., Török, Z., Páli, T., Balogh, G., Kovács, E., Nádasdi, L., Benkö, S., Joó, F., Vigh, L. (1998). Membrane physical state controls the signaling mechanism of the heat shock response in *Synechocystis* PCC 6803: identification of hsp17 as a "fluidity gene". *Proceedings of the National Academy of Sciences U S A*, 95(7), 3513-3518.

Hsieh, T.C., Wang, Z., Deng, H., & Wu, J. M. (2008). Identification of glutathione sulfotransferase-pi (GSTP1) as a new resveratrol targeting protein (RTP) and studies of resveratrol-responsive protein changes by resveratrol affinity chromatography. *Anticancer Research*, 28(1A), 29-36.

Hunter-Lavin, C., Davies, E. L., Bacelar, M. M., Marshall, M. J., Andrew, S. M., Williams, J. H. (2004). Hsp70 release from peripheral blood mononuclear cells. *Biochemical & Biophysical Research Communications*, 324(2), 511-517.

Jang, M., Cai, L., Udeani, G. O., Slowing, K. V., Thomas, C. F., Beecher, C. W., Fong, H. H., Farnsworth, N. R., Kinghorn, A. D., Mehta, R. G., Moon, R. C., & Pezzuto, J. M. (1997). Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science*, 275(5297), 218-20.

Jazirehi, A. R., & Bonavida, B. (2004). Resveratrol modifies the expression of apoptotic regulatory proteins and sensitizes non-Hodgkin's lymphoma and multiple myeloma cell lines to paclitaxel-induced apoptosis. *Molecular Cancer Therapeutics*, 3(1), 71-84.

Johnstone, R. W., Ruefli, A. A., & Lowe, S. W. (2002). Apoptosis: a link between cancer genetics and chemotherapy. *Cell*, 108(2), 153-164.

Jolly, C., & Morimoto, R. I. (2000). Role of the heat shock response and molecular chaperones in oncogenesis and cell death. *Journal of the National Cancer Institute*, 92(19), 1564-1572.

Jones, A. W., & Holmgren, P. (2003). Comparison of blood-ethanol concentration in deaths attributed to acute alcohol poisoning and chronic alcoholism. *Journal of Forensic Science*, 48(4), 874-879.

Khar, A., Ali, A. M., Pardhasaradhi, B. V., Varalakshmi, C. H., Anjum, R., & Kumari, A. L. (2001). Induction of stress response renders human tumor cell lines resistant to curcumin-mediated apoptosis: role of reactive oxygen intermediates. *Cell Stress Chaperones*, 6(4), 368-376.

Kalra, N., Roy, P., Prasad, S., & Shukla, Y. (2008). Resveratrol induces apoptosis involving mitochondrial pathways in mouse skin tumorigenesis. *Life Sciences*, 82, 348–358.

Kampinga, H. H., Hageman, J., Vos, M. J., Kubota, H., Tanguay, R. M., Bruford, E. A., Cheetham, M. E., Chen, B., & Hightower, L. E. (2009). Guidelines for the nomenclature of the human heat shock proteins. *Cell Stress Chaperones*, 14(1), 105-111.

Kubota, T., Uemura, Y., Kobayashi, M., & Taguchi, H. (2003). Combined effects of resveratrol and paclitaxel on lung cancer cells. *Anticancer Research*, 23(5A), 4039-4046.

Kundu, J. K., & Surh, Y. J. (2008). Cancer chemopreventive and therapeutic potential of resveratrol: mechanistic perspectives. *Cancer Letters*, 269(2), 243-61.

Lacour, S., Micheau, O., Hammann, A., Drouineaud, V., Tschopp, J., Solary, E., & Dimanche-Boitrel, M. T. (2003). Chemotherapy enhances TNF-related apoptosis-inducing ligand DISC assembly in HT29 human colon cancer cells. *Oncogene*, 22(12), 1807-1816.

Lançon, A., Delmas, D., Osman, H., Thénot, J. P., Jannin, B., & Latruffe, N. (2004). Human hepatic cell uptake of resveratrol: involvement of both passive diffusion and carrier-mediated process. *Biochemical & Biophysical Research Communications*, 316(4), 1132-1137.

Lanneau, D., Brunet, M., Frisan, E., Solary, E., Fontenay, M., & Garrido, C. (2008). Heat shock proteins: essential proteins for apoptosis. *Journal of Cellular & Molecular Medicine*, 12(3), 743-761.

Lin, M. T., Yen, M. L., Lin, C. Y., & Kuo, M. L. (2003). Inhibition of vascular endothelial growth factor-induced angiogenesis by resveratrol through interruption of Src dependent vascular endothelial cadherin tyrosine phosphorylation. *Molecular Pharmacology*, 64, 1029-1036.



Manna, S. K., Mukhopadhyay, A., & Aggarwal, B. B. (2000). Resveratrol suppresses TNF-induced activation of nuclear transcription factors NF- $\kappa$ B, activator protein-1, and apoptosis: potential role of reactive oxygen intermediates and lipid peroxidation. *The Journal of Immunology*, 164(12), 6509-6519.

Micheau, O., Solary, E., Hammann, A., Martin, F., & Dimanche-Boitrel, M. T. (1997). Sensitization of cancer cells treated with cytotoxic drugs to fas-mediated cytotoxicity. *Journal of the National Cancer Institute*, 89(11), 783-789.

Moulin, M., & Arrigo, A-P. (2006). Long lasting heat shock stimulation of TRAIL-induced apoptosis in transformed T lymphocytes. *Experimental Cell Research*, 312(10), 1765-1784.

Multhoff, G. (2007). Heat shock protein 70 (Hsp70): Membrane location, export and immunological relevance. *Methods*, 43(3), 229-237.

Nagy, E., Balogi, Z., Gombos, I., Akerfelt, M., Björkbom, A., Balogh, G., Török, Z., Maslyanko, A., Fiszer-Kierzkowska, A., Lisowska, K., Slotte, P. J., Sistonen, L., Horváth, I., & Vigh, L. (2007). Hyperfluidization-coupled membrane microdomain reorganization is linked to activation of the heat shock response in a murine melanoma cell line. *Proceedings of the National Academy of Sciences of the United States of America*, 104(19), 7945-7950.

Nourissat, P., Travert, M., Chevanne, M., Tekpli, X., Rebillard, A., Le Moigne-Müller, G., Rissel, M., Cillard, J., Dimanche-Boitrel, M. T., Lagadic-Gossmann, D., & Sergent, O. (2008). Ethanol induces oxidative stress in primary rat hepatocytes through the early involvement of lipid raft clustering. *Hepatology*, 47(1), 59-70.

Park, J. W., Choi, Y. J., Suh, S. I., Baek, W. K., Suh, M. H., Jin, I. N., Min, D. S., Woo, J. H., Chang, J. S., Passaniti, A., Lee, Y. H., & Kwon, T. K. (2001). Bcl-2 overexpression attenuates resveratrol-induced apoptosis in U937 cells by inhibition of caspase-3 activity. *Carcinogenesis*, 22(10), 1633-1639.

Psahoulia, F. H., Drosopoulos, K. G., Doubravska, L., Andera, L., & Pintzas, A. (2007). Quercetin enhances TRAIL-mediated apoptosis in colon cancer cells by inducing the accumulation of death receptors in lipid rafts. *Molecular Cancer Therapeutics*, 6(9), 2591-2599.

Putics, A., Végh, E. M., Csermely, P., & Söti, C. (2008). Resveratrol induces the heat-shock response and protects human cells from severe heat stress. *Antioxidants & Redox Signaling*, 10(1), 65-75.

Rashmi, R., Kumar, S., & Karunagaran, D. (2004). Ectopic expression of Hsp70 confers resistance and silencing its expression sensitizes human colon cancer cells to curcumin-induced apoptosis. *Carcinogenesis*, 25(2), 179-187.

Schmitt, E., Gehrmann, M., Brunet, M., Multhoff, G., & Garrido, C. (2007). Intracellular and extracellular functions of heat shock proteins: repercussions in cancer therapy. *Journal of Leukocyte Biology*, 81(1), 15-27.

Signorelli, P., & Ghidoni, R. (2005). Resveratrol as an anticancer nutrient: molecular basis, open questions and promises. *Journal of Nutritional Biochemistry*, 16(8), 449-466.

Soleas, G. J., Grass, L., Josephy, P. D., Goldberg, D. M., & Diamandis, E. P. (2002). A comparison of the anticarcinogenic properties of four red wine polyphenols. *Clinical Biochemistry*, 35(2), 119-124.

Sőti, C., Nagy, E., Giricz, Z., Vigh, L., Csermely, P., & Ferdinandy, P. (2005). Heat shock proteins as emerging therapeutic targets. *British Journal of Pharmacology*, 146(6), 769-780.

Srivastava, R., Ratheesh, A., Gude, R. K., Rao, K. V., Panda, D., & Subrahmanyam, G. (2005). Resveratrol inhibits type II phosphatidylinositol 4-kinase: a key component in pathways of phosphoinositide turn over. *Biochemical Pharmacology*, 70(7), 1048-1055.

Su, J. L., Lin, M. T., Hong, C. C., Chang, C. C., Shiah, S. G., Wu, C. W., Chen, S. T., Chau, Y. P., & Kuo, M. L. (2005). Resveratrol induces FasL-related apoptosis through Cdc42 activation of ASK1/JNK-dependent signaling pathway in human leukemia HL-60 cells. *Carcinogenesis*, 26(1), 1-10.

Sun, Z. J., Pan, C. E., Liu, H.S., & Wang, G. J. (2002). Anti-hepatoma activity of resveratrol in vitro. *World Journal of Gastroenterology*, 8(1), 79-81.

Surh, Y. J., Hurh, Y. J., Kang, J. Y., Lee, E., Kong, G., & Lee, S. J. (1999). Resveratrol, an antioxidant present in red wine, induces apoptosis in human promyelocytic leukemia (HL-60) cells. *Cancer Letters*, 140(1-2), 1-10.

Szabo, G., Dolganiuc, A., Dai, Q., & Pruett, S. B. (2007). TLR4, ethanol, and lipid rafts: a new mechanism of ethanol action with implications for other receptor-mediated effects. *Journal of Immunology*, 178(3), 1243-1249.

Vaculová, A., Hofmanová, J., Soucek, K., Andera, L., & Kozubík, A. (2004). Ethanol acts as a potent agent sensitizing colon cancer cells to the TRAIL-induced apoptosis. *FEBS Letters*, 577(1-2), 309-313.

Vigh, L., Horváth, I., Maresca, B., & Harwood, J. L. (2007). Can the stress protein response be controlled by 'membrane-lipid therapy'? *Trends in Biochemical Sciences*, 32(8), 357-363.

Vonghia, L., Leggio, L., Ferrulli, A., Bertini, M., Gasbarrini, G., & Addolorato, G. (2008). Acute alcohol intoxication. *European Journal of Internal Medicine*, 19(8), 561-567.

Wang, Y., Wang, B., Cheng, J., Yang, L., Liu, Z. L., Balan, K., Pantazis, P., Wyche, J. H., & Han, Z. (2005). FADD-dependent apoptosis induction in Jurkat leukemia T-cells by the resveratrol analogue, 3,4,5-trihydroxy-trans-stilbene. *Biochemical Pharmacology*, 69(2), 249-254.

Wenzel, E., & Somoza, V. (2005). Metabolism and bioavailability of trans-resveratrol. *Molecular Nutrition & Food Research*, 49(5), 472-481.

Wiseman, M. (2008). The Second World Cancer Research Fund/American Institute for Cancer Research Expert Report. Food, Nutrition, Physical Activity, and the Prevention of Cancer: A Global Perspective. *Proceedings of the Nutrition Society*, 67, 253-256.

Zoberi, I., Bradbury, C. M., Curry, H. A., Bisht, K. S., Goswami, P. C., Roti Roti, J. L., & Gius, D. (2002). Radiosensitizing and anti-proliferative effects of resveratrol in two human cervical tumor cell lines. *Cancer Letters*, 175(2), 165-173.